# PRELIMINARY REPORT ON THE ENHANCED ABSORBEFACIENT EFFECT OF THAM ON NASAL PEPTIDE PHARMACEUTICAL FORMULATIONS *(VIVO* STUDIES)

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## Introduction

Several scientific publications have recently reported an increasing interest for the administration of pharmaceutical formulations containing peptide compounds through the nasal mucosa.
Due to the limited absorption rate of peptides through this administration route, the main interest of scientists during the most recent years was to enhance the absorption rate of the drug product by adopting a suitable absorbefacient agent.
Nevertheless, published documents have neither considered nor reported any influence of enhancer concentration on the drug absorption profile.

### Scope

A study was therefore designed to support the absorbefacient effect of THAM in pharmaceutical nasal formulations containing known peptides, such as desmopressin and insulin.

The study has been divided in two parts, one related to desmopressin nasal formulation and the second to insulin nasal preparation.

## Desmopressin

### *Materials*

The following desmopressin nasal spray preparations were investigated and used as testing material, as shown in Table . 1.

**Table n. 1**

Quali-quantitative compositions of Nasal Formulations of desmopressin nasal spray solution to be tested.

| 1.0 ml of nasal spray solution containing: | NASAL FORMULATION N°. | | | | | | |
|---|---|---|---|---|---|---|---|
| | **0** (1-6) | **1** | **2** | **3** | **4** | **5** | **6** |
| Peptide and Ingredients: | mg | mg | mg | mg | mg | mg | mg |
| Desmopressin acetate (DDAVP) (equivalent to desmopressin) | 0.1126 (0.1000) | | | | | | |
| THAM | 0.0 | 1.0 | 2.0 | 4.4 | 10.0 | 20.0 | 30.0 |
| Citric acid | 6.28 | | | | | | |
| Methyl p-hydroxybenzoate | 0.27 | | | | | | |
| Propyl p-hydroxybenzoate | 0.03 | | | | | | |
| Distilled water q.s. to ml | 1.00 | | | | | | |

***Subjects and methods***

Administered single dose of 20 μg (2x 10 μg) desmopressin, study design (4 healthy subjects), blood sampling and analytical procedures were carried out as described in the published literature (1).

Each desmopressin Nasal Formulation (as spray solution) was individually administered by intranasal administration route by means of a device (dosing pump and nasal applicator), such as those commonly available on the market. Each actuation volume dispensing 0.10 ml of nasal solution, corresponding to 20 μg desmopressin.

***Pharmacodynamic and pharmacokinetic calculations***

For each tested Nasal Formulation from N°. 0 (1-6) to 6, a blood sample was collected at 45 minutes after a single dose (20 μg)

administration by nasal route , being the Maximum level of desmopressin plasma concentration reached in about 45 minutes, as described in the published literature (1).

The individual desmopressin Maximum plasma concentrations and the mean (arithmetic mean and S.D.) plasma desmopressin concentrations are reported in Table n. 2.

**Table n. 2**

Individual desmopressin Maximum plasma concentrations and arithmetic mean and S.D.

| Desmopressin Maximum plasma concentrations (pg/ml) | NASAL FORMULATION N°. | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0 (1-6) | 1 | 2 | 3 | 4 | 5 | 6 |
| **Subject 1** | 8.9 | 23.3 | 28.4 | 45.7 | 47.3 | 31.5 | 29.3 |
| **Subject 2** | 19.1 | 13.9 | 17.8 | 32.3 | 36.7 | 41.4 | 41.8 |
| **Subject 3** | 9.6 | 25.1 | 16.8 | 44.1 | 47.9 | 29.7 | 29.8 |
| **Subject 4** | 18.2 | 12.6 | 26.5 | 34.4 | 36.6 | 38.9 | 38.5 |
| **Mean** | 13.9 | 18.7 | 22.4 | 39.1 | 42.1 | 35.4 | 34.8 |
| **S.D. ±** | 5.45 | 6.39 | 5.93 | 6.75 | 6.33 | 5.66 | 6.27 |

The significant absorbefacient effect of THAM in the tested Nasal Formulations is shown by the arithmetic mean Maximum plasma desmopressin concentrations (pg/ml) at 45 minutes following to a single dose nasal administration (20 μg) of each Nasal Formulation and the results are reported in Table n. 3.

**Table n. 3**

Arithmetic mean of Maximum plasma desmopressin concentrations (pg/ml).

| PARAMETERS | NASAL FORMULATION N°. | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0 (1-6) | 1 | 2 | 3 | 4 | 5 | 6 |
| **THAM concentrations (mg/ml)** | 0.0 | 1.0 | 2.0 | 4.4 | 10.0 | 20.0 | 30.0 |
| **Arithmetic mean of Desmopressin Maximum plasma concentrations (pg/ml)** | 13.9 | 18.7 | 22.4 | 39.1 | 42.1 | 35.4 | 34.8 |

***Discussion and Conclusion***

In this study the absorbefacient effect of THAM in pharmaceutical Nasal Formulations containing desmopressin has been investigated in relation to the absorption rate of the peptide product.

The experimental conditions and determinations of the case study have been carried out by partially following the applicable procedures, such as those described in published literature (1).

The Maximum desmopressin plasma concentrations (pg/ml) have been determined at the predictable maximum peak of 45 minutes following to a single dose nasal administration of 20 μg of each Nasal Formulation under testing.

The results of the above study, clearly demonstrate that THAM exhibits an enhanced absorbefacient effect on the peptide desmopressin contained in Nasal Formulations.

## Insulin (human recombinant)

***Materials***

Insulin (human recombinant) nasal spray preparations were investigated and used as testing material, as shown in Table n. 4.

**Table n. 4**

Quali-quantitative compositions of Nasal Formulations of Insulin (human recombinant) nasal spray solution to be tested.

| 1.0 ml of nasal spray solution containing: | NASAL FORMULATION N°. | | | | | | |
|---|---|---|---|---|---|---|---|
| | **0** (7-12) | **7** | **8** | **9** | **10** | **11** | **12** |
| Peptide and ingredients: | mg | mg | mg | mg | mg | mg | mg |
| Insulin human recombinant (U) | 500 U | | | | | | |
| THAM | 0.0 | 1.5 | 3.0 | 5.8 | 12.0 | 24.0 | 30.0 |
| Hydrochloric acid | 2.9 | | | | | | |
| Methyl p-hydroxybenzoate | 1.2 | | | | | | |
| Distilled water q.s. to ml | 1.00 | | | | | | |

***Subjects and methods***

Administered single dose of 50 U of insulin per subject, study design (6 healthy subjects), blood sampling and analytical procedures were carried out with applicable methods as those described in the published literature (2) (3) (4). Each insulin Nasal Formulation (as spray solution) was individually administered by intranasal administration route by means of a device (dosing pump and nasal applicator), such as those commonly available on the market. Each actuation volume is dispensing a volume of 0.10 ml of nasal solution, corresponding to 50 U of insulin human recombinant.

***Pharmacodynamic and pharmacokinetic calculations***

For each tested Nasal Formulation from N°. 0 (7-12) to 12, a blood sample was collected at 20 minutes after a single dose (50 U) administration to each subject by nasal route, being the Maximum level of insulin plasma concentration reached in about 20 minutes, as described in the published literature (2). The individual insulin Maximum plasma concentrations and the mean (arithmetic mean and S.D.) plasma concentrations are reported in Table n. 5.

**Table n. 5**

Individual insulin human recombinant Maximum plasma concentrations and arithmetic mean and S.D.

| Insulin Maximum plasma concentrations (μU/ml) | NASAL FORMULATION N°. | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0 (7-12) | 7 | 8 | 9 | 10 | 11 | 12 |
| **Subject 5** | 8.9 | 12.3 | 19.1 | 53.7 | 43.8 | 36.9 | 29.1 |
| **Subject 6** | 20.7 | 25.4 | 29.4 | 53.2 | 51.1 | 44.5 | 35.7 |
| **Subject 7** | 11.0 | 14.3 | 17.8 | 38.3 | 39.4 | 33.6 | 41.2 |
| **Subject 8** | 19.1 | 24.7 | 23.0 | 46.7 | 52.3 | 45.6 | 36.1 |
| **Subject 9** | 10.5 | 19.6 | 28.9 | 54.1 | 41.7 | 32.3 | 44.8 |
| **Subject 10** | 9.3 | 24.5 | 18.6 | 44.5 | 50.6 | 41.8 | 34.9 |
| **Mean** | 13.2 | 20.1 | 22.8 | 48.4 | 46.5 | 39.1 | 36.9 |
| **S.D. ±** | 5.23 | 5.72 | 5.24 | 6.38 | 5.52 | 5.66 | 5.44 |

**Table n. 6**

Arithmetic mean of Maximum plasma insulin concentrations (μU/ml).

| PARAMETERS | NASAL FORMULATION N°. | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0 (7-12) | 7 | 8 | 9 | 10 | 11 | 12 |
| **THAM concentrations (mg/ml)** | 0.0 | 1.5 | 3.0 | 5.8 | 12.0 | 24.0 | 30.0 |
| **Arithmetic mean Maximum plasma concentrations of Insulin (μU /ml)** | 13.2 | 20.1 | 22.8 | 48.4 | 46.5 | 39.1 | 36.9 |

***Discussion and Conclusion***

In this study the absorbefacient effect of THAM in pharmaceutical Nasal Formulations containing insulin (human recombinant) has been investigated in relation to the absorption rate of the peptide.

The experimental conditions and determinations of the case study have been carried out by following, when applicable, the procedures, such as those described in published literature (2) (3).

The Maximum insulin plasma concentrations (μU/ml) have been determined at the predictable maximum peak of 20 minutes following to a single dose nasal administration of 50 U of each Nasal Formulation under testing.

The results of the above study, demonstrate the enhanced absorbefacient effect of THAM on the peptide insulin (human recombinant) contained in Nasal Formulations.

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Published litterature:

(1) Eller N. , Kollenz CJ. , Hitzenberger G. , A comparative study of pharmacodynamics and bioavailability of 2 different desmopressin nasal sprays. *Int J Clin Pharmacol Ther.* , 1998, vol. 36 (3), 139-45

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(3) Valensi P. , Zirinis P. , Nicolas P. , Perret G. , Sandre-Banon D. , Attali JR. , Effect of insulin concentration on bioavailability during nasal spray administration. *Pathol Biol (Paris)*, 1996, vol. 44 (4), 235-40

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# PUBLISHED LITERATURE

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# A comparative study of pharmacodynamics and bioavailability of 2 different desmopressin nasal sprays

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**Abstract.** The antidiuretic effect and pharmacokinetics were investigated in 16 healthy, male overhydrated volunteers after intranasal administration of 20 µg desmopressin. The antidiuretic activity was measured by determination of urine osmolality and diuresis every 15 minutes over a period of 8 hours. Both study preparations were equally effective regarding a rapid onset of activity and a highly reproducible magnitude of effect. Urine osmolalities, analyzed as area under the time curve ($AUC_{osm}$) and maximum urine osmolalities were similar for both nasal sprays. Urine volume, analyzed as area under the time curve, was raised after treatment with the test preparation. Bioequivalence was assessed for the primary criterion $AUC_{osm}$ by a calculated mean ratio (test/reference) of 102.8% and a 90% confidence interval ranging from 95.4% to 110.8%. Plasma levels of desmopressin, measured by a specific and sensitive radio-immunoassay method, were already detectable 20 minutes after administration. The mean time curves were parallel at different concentration levels. The maximum desmopressin plasma concentrations of both preparations were comparable, showing high interindividual variability. The times of reaching maximum plasma concentrations were similar. Desmopressin bioavailability was increased after treatment with the test preparation (mean ratio of 130.8% and a 90% confidence interval ranging from 109.9% to 155.7%). Both preparations showed a pronounced biological effect with similarly raised urine osmolalities. The detected differences in bioavailability seem to have no direct correlation to the biological response.

**Key words:** 1-deamino-8-d-arginine vasopressin – desmopressin intranasally – urine osmolality – urine volume – desmopressin pharmacokinetics

## Introduction

With the discovery of the chemical structure of the neurohypophyseal nonapeptide hormone vasopressin [Du Vigneaud et al. 1954] and its subsequent laboratory synthesis, it became possible to synthesize structural analogues. Desmopressin (dDAVP; 1-deamino-8-D-arginine vasopressin), one analogue synthesized in the 1960s by the Czechoslovak Academy of Sciences, proved to be a highly selective antidiuretic agent. Two structural changes distinguish desmopressin from the hormone arginine-vasopressin:

- deamination of cysteine at the N-terminal of the molecule, and
- substitution of L-arginine at position 8 with the enantiomer D-arginine [Vilhardt 1990].

These changes result in an increased antidiuretic effect, a prolonged biological half-time, and diminish pressor activity.

Antidiuretic efficacy after intranasal, buccal, gastrointestinal, and parenteral application has been proven [Curtis and Donovan 1979, Harris et al. 1987, Monson et al. 1974, Richardson and Robinson 1985, Williams et al. 1986]. The efficacy (power and duration) is dose-dependent. In order to achieve the same antidiuretic effect compared to intranasal application 10 – 20 times higher doses have to be administered orally. Because of its potent antidiuretic effect, the missing pressor activity and the minimal side-effects, desmopressin is the drug of choice for the treatment of central idiopathic or secondary diabetes insipidus [Andersson and Arner 1972, Becker and Foley 1978]. Furthermore, dDAVP is used in enuresis [Dimson 1977, Pedersen et al. 1985], for renal concentration tests [Delin et al. 1978], for nocturia management in multiple sclerosis [Valiquette et al. 1992] and as an activator and mobilizer of factor VIII reserves in hemophilia A and Willebrand's factor in Willebrand Jürgens syndrome [Mannucci et al. 1977, Vilhardt et al. 1980].

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Recent studies have demonstrated the effectiveness of desmopressin treatment via the intranasal route of administration. The purpose of the present study was to compare a new desmopressin nasal spray with the established innovator reference preparation and to meet requirements for registration in the EU. A study was therefore designed to compare the effect profiles and plasma dDAVP profiles obtained after administration of 20 µg of desmopressin given intranasally in 16 healthy male volunteers.

## Subjects, material, and methods

### *Study design*

The study was carried out in 16 healthy male volunteers in an open, randomized 2-way crossover design with a single-dose administration of 20 µg desmopressin intranasally. After hospitalization and fasting for 12 hours (liquid intake ad libitum) a standard breakfast was served, and blood pressure and heart rate were taken. Water loading (1.5% of body weight) with tap water followed. As soon as the 15-minute urine volume reached at least 150 ml, 20 µg (2 × 10 µg) of desmopressin were administered in conformity with the randomization list. The spray pump was actuated 3 times before administration, and the container weighed before and after administration. The test and the reference preparations were administered to all subjects by the same person. After urine collection the volunteers had to drink an amount of tap water that equalled the urine passed. Between the test runs a wash-out period of at least 1 week was prescribed. Standard lunch and dinner were served 5 and 10 hours after administration.

### *Subjects*

Sixteen male volunteers with an average age of 29.5 years (SD = 4.1), an average weight of 76.1 kg (SD = 7.6) and an average height of 180.2 cm (SD = 6.3) took part in the study. On the basis of medical and laboratory examinations they were judged to be healthy, and they met the inclusion criteria. The study was conducted in accordance with the Declaration of Helsinki (Venice Revision 1983) and the principles of GCP. The protocol was approved by the Ethics Committee "Österreichische Arbeitsgemeinschaft für klinische Pharmakologie – Ethikkommission". The volunteers confirmed their written consent to enrolment after detailed information had been provided to them.

### *Material*

The following 2 desmopressin nasal sprays were investigated:

A) desmopressin test nasal spray with the composition of 0.1 mg desmopressin acetate and 5.0 mg chlorobutanol per ml (Gebro Broschek GmbH, Fieberbrunn/Austria).

B) reference nasal spray with the composition of 0.1 mg desmopressin acetate and 5.0 mg chlorobutanol per ml (Minirin 0.1 mg/ml Nasenspray, Ferring AB, Malmö, Sweden).

Both preparations release 10 µg desmopressin-acetate per spray blast.

### *Sampling*

#### Urine samples

After water-loading (1.5% of body weight) with tap water urine samples were taken every 15 minutes. As soon as the 15-minute urine volume reached 150 ml, the study preparations were administered. From the time of application on urine was collected every 15 minutes for 8 hours. Urine volume and osmolality were measured for all urine samples.

#### Blood samples

Blood (10 ml) was collected in heparinized polypropylene tubes from a cubital vein before administration of the study preparations and 20, 40, 60, 90, 120, 180, 240, 360, and 480 minutes after administration. The first drops were discarded, and plasma was frozen at –20° C until analysis.

#### Analytical procedures

Urine osmolality was determined by a cryoscopic method (Osmomat 030, Gonotec, Berlin, Germany) immediately after collection.

Plasma was stored at –20° C for measurement of dDAVP by radio-immunoassay (RIA). After extraction of 2 ml samples with 200 µl 2 M sodium dihydrogen phosphate on Chromabond C 18 ec cartridges, eluates were frozen at –80° C for at least 1 h and lyophilized. The samples were reconstituted in 450 µl RIA buffer, and dDAVP was measured with a highly specific and sensitive RIA using 400 µl sample or standard, 50 µl [$^{125}$I]dDAVP (6,000 cpm ± 10%) and 50 µl specific dDAVP antiserum. Incubation was carried out for 16 – 24 hours at 4° C, and antibody-bound tracer was separated from the free fraction by charcoal separation. The lower limit of quantification was 3.5 pg/ml.

#### Pharmacodynamic and pharmacokinetic calculations; statistical analysis

The criterion area under the urine osmolality time curve from 0 to 480 minutes ($AUC_{osm}$ in Osm × min/kg)

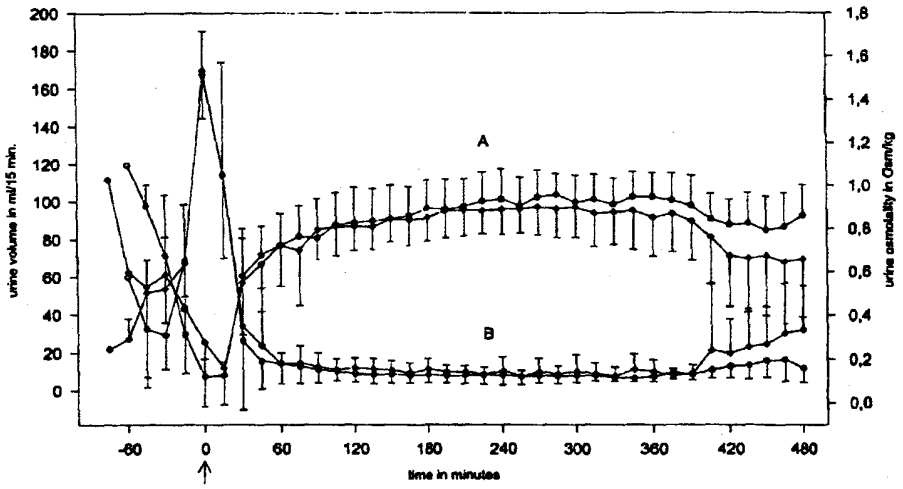


*Fig. 1* Comparative time courses of mean urine osmolality (A) and urine volume (B) time data after intranasal administration of 20 µg of desmopressin (administration marked by arrow) as (O) test preparation or (◆) reference preparation (arithmetic means ± SD, sample size n = 16).

*Table 1* Areas under the urine osmolality time curves 0 – 480 minutes ($AUC_{osm}$), areas under the urine volume time curves 0 – 480 minutes ($AUC_{vol}$) and maximum urine osmolalities following administration of 20 µg desmopressin intranasally.

| | Area under the urine osmolality time curve $AUC_{osm}$ | | Area under the urine volume time curve $AUC_{vol}$ | | Maximum urine osmolality | |
|---|---|---|---|---|---|---|
| | Test | Reference | Test | Reference | Test | Reference |
| Arith. mean | 384.855 | 372.107 | 7,796.8 | 9,461.2 | 1.016 | 0.968 |
| SD | 57.775 | 61.679 | 2,360.8 | 3,526.7 | 0.101 | 0.112 |
| Geom. mean | 378.764 | 368.366 | 7,445.0 | 8,803.2 | 1.008 | 0.965 |
| Mean log transf. | 2.578 | 2.566 | 3.872 | 3.945 | 0.003 | –0.015 |
| Minimum | 261.210 | 250.635 | 4,432.5 | 3,997.5 | 0.825 | 0.724 |
| Maximum | 455.317 | 460.935 | 1,1707.5 | 16,155.0 | 1.174 | 1.142 |
| N | 16 | 16 | 16 | 16 | 16 | 16 |

Areas calculated by the trapezoidal integration rule; arithmetic mean (arith. mean), standard deviation (SD), geometric mean (geom. mean), mean log-transformed, minimum, maximum, and sample size (N); $AUC_{osm}$ in Osm × min/kg, $AUC_{vol}$ in ml × min, maximum urine osmolality in Osm/kg

was defined as primary parameter. In addition, the parameters area under the urine volume time curve from 0 to 480 minutes ($AUC_{vol}$ in ml × min) and the maximal increase in urine osmolality (Osm/kg per 15 minutes) were analyzed. Desmopressin plasma concentrations were compared by means of the pharmacokinetic parameters $AUC_{0\text{-}480min}$, $C_{max}$, and $t_{max}$. Local tolerance as well as adverse events were documented.

The areas were calculated by the trapezoidal integration rule. The other parameters were determined from the individual time curves. The statistical analysis was based on a crossover analysis with testing for effects of carryover, treatment, and period (Software package "Testimate Vers. 5.2", IDV-Versuchsplanung Gauting/München). After logarithmic transformation of the raw data, the 2 one-sided t-tests procedure was used to calculate mean ratios (test/reference) with corresponding 90% confidence intervals. As commonly accepted, a range for equivalence of 80% – 125% was defined.

## Results

### *Antidiuretic effect*

The antidiuretic response following administration of the 2 desmopressin nasal sprays is shown in Figure 1, which combines mean urine osmolality and urine volume time curves. Both preparations induce rapid, almost identical antidiuresis (start volumes almost identical), which starts 15 – 30 minutes after administration. For both preparations, an antidiuresis of < 25 ml per min occurs after 45 minutes. After 1 hour, the mean urine flow rate is below 15 ml for both preparations. Urine osmolality increases rapidly after 15 – 30 minutes and reaches a mean value of 0.72 Osm/kg for both nasal sprays. In the last 90 minutes the antidiuretic effect is more pronounced under treatment with the test preparation. This is due to a more rapidly declining effect in 4 subjects treated with the reference preparation.

*Table 2* Statistical evaluation (2 one-sided t-tests, log-transformed data) for the criteria $AUC_{osm}$, $AUC_{vol}$, maximum urine osmolality, $AUC_{0\text{-}480min}$, and $C_{max}$, mean ratios with corresponding 90% confidence limits, p values for carryover-, period- and treatment-effects.

| | Mean ratio | 90% CI | Carryover effect | Period effect | Treatment effect |
|---|---|---|---|---|---|
| $AUC_{osm}$ | 102.8 | 95.4 – 110.8 | 0.934 | 0.168 | 0.523 |
| $AUC_{vol}$ | 84.6 | 70.1 – 102.0 | 0.729 | 0.908 | 0.138 |
| Maximum urine osmolality | 104.4 | 98.6 – 110.5 | 0.898 | 0.090 | 0.205 |
| $AUC_{0\text{-}480min}$ | 130.8 | 109.9 – 155.7 | 0.368 | 0.278 | 0.017 |
| $C_{max}$ | 114.0 | 94.4 – 137.8 | 0.090 | 0.545 | 0.242 |

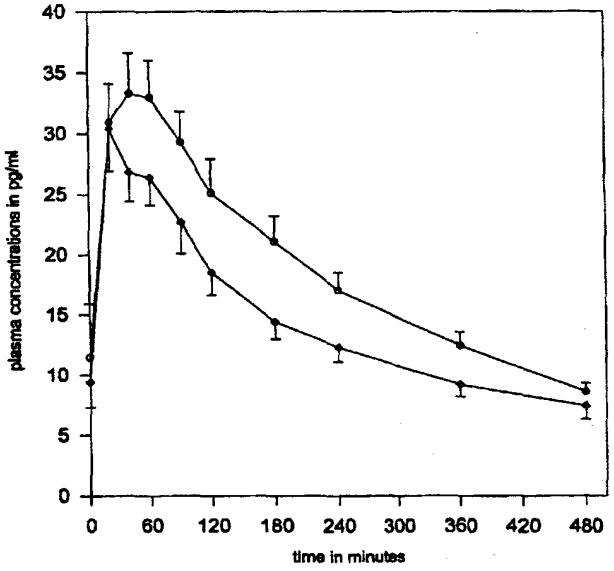


*Fig. 2* Comparative time courses of mean dDAVP plasma concentrations following administration of 20 µg of desmopressin (○) as test preparation or (◆) reference preparation (pg/ml, arithmetic means ± SEM).

$AUC_{osm}$ values, which indicate the urine concentrating potency of the desmopressin sprays, are presented in Table 1. Regarding this criterion both preparations are comparable with arithmetic mean values (± SD) of 384.855 ± 57.775 Osm × min/kg for the test preparation and 372.107 ± 61.679 Osm × min/kg for the reference preparation. With a calculated mean ratio of 102.8% and a corresponding 90% confidence interval ranging from 95.4% to 110.8% both desmopressin sprays are bioequivalent (Table 2).

Results of the antidiuresis parameter $AUC_{vol}$ are presented in Table 1. Mean values (± SD) of 7,796.8 ± 2,360.8 ml × min for the test preparation and of 9,461.2 ± 3,526.7 ml × min for the reference preparation were calculated. The mean ratio of 84.6% with a corresponding 90% confidence interval ranging from 70.1% to 102.0% indicates a slightly more pronounced antidiuretic response after treatment with the test preparation (Table 2). As shown in Figure 1 this result is mainly due to a difference in the last 90 minutes, when urine volume increased in a more pronounced manner with the reference preparation.

Individual maximum urine osmolalities are presented in Table 1. The values observed for the test preparation range from 0.825 to 1.174 Osm/kg (arithmetic mean SD: 1.016 ± 0.101), and from 0.724 to 1.142 (arithmetic mean SD: 0.968 ± 0.112 Osm/kg) for the reference preparation. The statistical analysis is presented in Table 2. Both desmopressin preparations are bioequivalent with a calculated mean ratio of 104.4% and a 90% confidence interval ranging from 98.6% to 110.5%.

### *Pharmacokinetics of dDAVP*

The mean (arithmetic mean and SD) plasma desmopressin concentration time curves are shown in Figure 2, pharmacokinetic characteristics are listed in Table 3. The mean time curves of both preparations are parallel on different concentration levels. Desmopressin is already detectable 20 minutes after administration. Maximum levels were reached after 46.3 minutes for the test preparation and after 43.1 minutes for the reference preparation. Maximum concentrations (arithmetic mean ± SD) of 38.6 ± 14.9 pg/ml (test preparation) and of 34.0 ± 13.4 pg/ml (reference preparation) were measured. For the characteristic $AUC_{0\text{-}480min}$ arithmetic means of 9,041.31 ± 2,873.42 pg × min/ml (test preparation) and of 6,929.03 ± 2,467.30 pg × min/ml (reference preparation) were calculated.

The statistical analysis indicates for both parameters increased desmopressin bioavailability after administration of the test preparation (Table 2). Mean ratios of 130.8% (90% CI: 109.9 – 155.7%) for $AUC_{0\text{-}480min}$ and of 114.0% (90% CI: 94.4% – 137.8%) for $C_{max}$ were calculated. Applying a 10% level of significance for pretests, as sometimes recommended, a positive carryover effect (p value of 0.090) for the parameter $C_{max}$ could not be excluded. Additionally a nonselective 2-group comparison (t-test with n = 8) using data only of period 1 was performed. A mean ratio of 150.8% with a 90% confidence interval ranging from 112.4% to 202.7% was calculated.

*Table 3* Pharmacokinetic data following administration of 20 µg desmopressin intranasally

| | Area under the dDAVP plasma time curve $AUC_{0\text{-}480min}$ | | Maximum dDAVP plasma concentration $C_{max}$ | | Time of maximum dDAVP plasma concentration $t_{max}$ | | Elimination half-life | |
|---|---|---|---|---|---|---|---|---|
| | Test | Reference | Test | Reference | Test | Reference | Test | Reference |
| Arith. mean | 9,041.3 | 6,926.0 | 38.6 | 34.0 | 46.25 | 43.13 | 3.8 | 3.8 |
| SD | 2,873.4 | 2,467.3 | 14.9 | 13.4 | 23.63 | 27.98 | 1.1 | 1.1 |
| Geom. mean | 8,501.7 | 6,498.4 | 35.4 | 31.1 | – | – | – | – |
| Mean log transf. | 3.930 | 3.813 | 1.549 | 1.492 | – | – | – | – |
| Minimum | 4,764.5 | 3,255.5 | 22.4 | 15.6 | 0 | 20 | 2.3 | 2.7 |
| Maximum | 15,351.5 | 11,414.0 | 74.0 | 59.5 | 90 | 90 | 6.7 | 6.7 |
| N | 16 | 16 | 16 | 16 | 16 | 16 | 16 | 16 |

Areas calculated by the trapezoidal integration rule, arithmetic mean (arith. mean), geometric mean (geom. mean), mean log-transformed, standard deviation (SD), minimum, maximum, and sample size (N), $AUC_{0\text{-}480min}$ in pg × min/ml, $C_{max}$ in pg/ml, $t_{max}$ in minutes, half-life in hours

*Drug safety*

In no case were irritations of the nasal mucosa observed. No drug-related adverse reactions were documented. No clinically relevant changes in the physical status, ECG, blood pressure, and heart rate were observed.

## Discussion

Numerous reports have documented desmopressin as the drug of choice in the treatment of cranial diabetes insipidus and nocturnal enuresis in both adults and children [Belmaker and Bleich 1986, Edwards et al. 1973, Fjellestad-Paulsen et al. 1987, Pedersen et al. 1985, Robinson 1976]. The long-lasting effect of desmopressin makes it possible for most patients to control their polyuria with 1 or 2 doses per day. The uterotonic activity of desmopressin is 10 times lower than that of vasopressin and the drug has been used in pregnant patients without any untoward effects on mother or fetus [Burrow et al. 1981, Oravec and Lichardus 1972, Vilhardt 1990].

In the case of nocturnal enuresis the primary rationale is that a single dose of the drug before bedtime will reduce urine formation over the next 8 – 10 hours, so that the micturition reflex is not triggered by a filled bladder [Vilhardt 1990].

In this study, conducted as an equivalence study, 2 desmopressin nasal sprays with identical desmopressin amount were investigated. As reference preparation a well documented desmopressin innovator nasal spray containing 0.1 mg/ml desmopressin was chosen. The study was conducted in water-loaded, healthy male volunteers. The study design was according to Harris et al. [1987]. Urine collections every 15 minutes for 8 hours provided a sufficiently detailed time course. Bioequivalence was proven according to the EC-Guideline [Note for Guidance: Investigation of bioavailability and bioequivalence 1991] on predefined criteria characterizing the pharmacodynamic and pharmacokinetic profiles of desmopressin.

Assessing objective variables (urine osmolality, desmopressin plasma concentrations) and difficulties by changing the nasal pump spray, which could change the pharmacokinetic and/or pharmacodynamic properties, the study was conducted as an open-label study.

Both desmopressin nasal sprays showed a marked antidiuretic effect in water-loaded healthy volunteers, which reached a maximum at 4.50 – 4.75 hours after administration. Maximum urine osmolalities ranged from 0.724 to 1.174 with comparable mean values for both preparations. The effect was still appreciable after 8 hours with indications for a prolonged antidiuretic effect after treatment with the test preparation. In summary, the mean time courses of both desmopressin preparations indicate a comparable urine concentrating effect with a similar biological response in the time of onset, magnitude, and duration of antidiuretic activity.

In this study no apparent differences were observed for the parameter $AUC_{osm}$. The tests for equivalence showed bioequivalence with a mean ratio of 102.8% and a 90% confidence interval ranging from 95.4% to 110.8%.

The analysis of the parameter $AUC_{vol}$, which combines urine flow rates over the entire observation period, missed the equivalence range with a 90% confidence interval ranging from 70.1% to 102.0%. However, this parameter is obviously susceptible to biased interpretation. Slight variations in the individual volume data result in relative high percent increases in the absolute values of this characteristic.

The maximum urine osmolality values were similar, indicating an equivalent maximal urine concentrating effect of the desmopressin nasal sprays tested. Bioequivalence was proven by the calculated mean ratio of 104.4% and the 90% confidence interval ranging from 98.6% to 110.5%.

The plasma profile of dDAVP showed a difference in plasma levels between the 2 nasal sprays. Desmopressin appeared in the plasma already 20 minutes after administration. Subsequently the concentration decreased following first-order kinetics for both study preparations. Peak plasma levels were measured after 43 minutes for the reference preparation and after 46 minutes for the test preparation. These data agree with data from studies in healthy volunteers and children with diabetes insipidus [Fjellestad-Paulsen et al. 1987] which showed $t_{max}$ values of 39 and 60 minutes. In the present study the maximum dDAVP plasma concentration following intranasal administration was slightly higher for the test preparation and lower compared to previously conducted studies where identical doses were given [Fjellestad-Paulsen et al. 1987, Tryding et al. 1987, Vilhardt et al. 1986]. A pretest could not exclude a carryover effect (p value of 0.090) for the parameter $C_{max}$. This quite insensitive test was not significant for a treatment- or period effect. This means that the treatment difference was different between the 2 study periods, where in the case of the parameter $C_{max}$ the test preparation was superior in the first period and inferior in the second period. As consequence a t-test using only data of period 1 as performed which confirmed this result (mean ratio of 150.8% for period 1). By testing this parameter with the defined 2 one-sided t-test procedure a mean ratio of 114.0% and a 90% confidence interval ranging from 94.4% to 137.8% was calculated. Being aware of a high interindividual variability for this parameter and the fact of inferiority of the test preparation in the second period the analysis showed superiority of the test preparation.

Regarding the parameter $AUC_{0\text{-}480min}$ dDAVP bioavailability was higher after administration of the test preparation. The statistical analysis showed a clear distinction between the 2 nasal sprays with a mean ratio of 130.8% and a 90% confidence interval ranging from 109.9% to 155.7%. It may be that the technique of intranasal administration may lead to large variation in the absorption of dDAVP.

It would be reasonable to assume that higher dDAVP plasma concentrations would result in more elevated urine osmolalities. This study and results of previous studies have shown that this is not the case [Fjellestad-Paulsen et al. 1987, Tryding et al. 1987]. The pharmacodynamic effect of both desmopressin nasal sprays lasts at least 7 hours. This effect lasts longer than the increase in plasma concentration, indicating that beside the wide interindividual variation it may be that even the lower plasma concentrations of dDAVP achieved are above the threshold concentration required and induce already maximum urinary concentration.

For the assessment of the clinical relevance of differences in dDAVP bioavailability one has to consider that the intranasal doses necessary vary in several studies and are not found to be related to age, the severity of polyuria, or to the body weight of the patient. A different metabolism and/or clearance of dDAVP may necessitate an individual adjustment of the dosage regimen [Aronson et al. 1973, Fjellestad-Paulsen et al. 1987].

## Conclusion

As the main purpose of the study was to compare the antidiuretic and urine concentrating effect, overhydrated subjects were investigated. Both preparations showed a distinct biological effect with similarly elevated urine osmolalities over the entire observation period and a good correlation between urine osmolality and urine volume. The detected differences in bioavailablity of dDAVP seem to have no direct correlation to the biological response.

**Acknowledgements**

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# PUBLISHED LITERATURE

(2)

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# An Effective Absorption Behavior of Insulin for Diabetic Treatment Following Intranasal Delivery Using Porous Spherical Calcium Carbonate in Monkeys and Healthy Human Volunteers

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## ABSTRACT

**Porous spherical calcium carbonate (PS-$CaCO_3$), in contrast to regular calcium carbonate ($CaCO_3$), which has a cuboidal particle shape, has a characteristic spherical particle shape with a large number of porous, sliver crystals. The effect of PS-$CaCO_3$ as a drug carrier on intranasal insulin absorption was investigated in cynomolgus monkeys and healthy human volunteers. Each insulin formulation (powder) containing PS-$CaCO_3$ or regular $CaCO_3$ was administered intranasally. Serum insulin and glucose levels after administration were evaluated. The insulin absorption after intranasal administration with each $CaCO_3$ was found to be much more rapid than that after subcutaneous administration. The serum insulin level after intranasal insulin delivery (16 U per monkey) with PS-$CaCO_3$ showed a higher $C_{max}$ (403.5 $\mu$U/mL) and shorter $T_{max}$ (0.167 h) when compared with regular $CaCO_3$. The serum glucose level reduction rate after intranasal delivery using PS-$CaCO_3$ was faster than that of regular $CaCO_3$, reflecting the difference in absorption rates. Following repeated intranasal administrations for 4 weeks in monkeys, no toxicity was observed even with a maximum insulin dose level of 25 U. Furthermore, the intranasal insulin absorption rate with PS-$CaCO_3$ in healthy humans was also observed to be considerably faster than that with regular $CaCO_3$. Effects of PS-$CaCO_3$ on a more effective absorption behavior of insulin were considered to be the result of a greater affinity between the nasal mucosa layer and PS-$CaCO_3$, which is closely related to its structural characteristics. Thus, intranasal insulin delivery using PS-$CaCO_3$ is thought to be a safe and highly available system enabling more effective insulin absorption behavior with the appearance of endogenous postprandial insulin secretion in healthy humans. We believe that our intranasal insulin delivery system enabling a rapid and short-acting pharmacological effect against postprandial hyperglycemia will be more beneficial than pulmonary insulin delivery systems in the treatment of diabetes.**

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## INTRODUCTION

DIABETIC PATIENTS usually require painful single or multiple daily injections of insulin, leading to low compliance of patients' medical regimens. To develop a promising insulin delivery system as an alternative to subcutaneous injection, a number of investigations into alternative routes of insulin delivery, including inhalation,[1] intranasal,[2] transdermal,[3] oral,[4] buccal,[5] and rectal[6] administration, have been performed.

Currently, major pharmaceutical companies are vigorously developing pulmonary insulin delivery systems and have advanced these to late stages in clinical trials.[7–9] However, the chronic pulmonary insulin delivery may include a concern about pulmonary fibrosis. The use of injectable insulin formulations (fast-acting products) for postprandial hyperglycemia often leads to a hypoglycemic side effect due to excessive duration of the blood insulin concentration.

It is reported that insulin administered intranasally shows rapid and short-term absorption, resulting in a rapid and short-acting pharmacological effect, and is likely to reproduce endogenous postprandial insulin secretion in healthy humans.[10] Intranasal insulin delivery should, therefore, lead to both a shortening of the onset of post-dosing pharmacological effects and the reduction of hypoglycemic side effects. Although most attempts at intranasal insulin delivery have achieved adequate absorbability by utilizing absorption enhancers,[11,12] these have not yet led to clinical use owing to possible toxicological effects. Yanagawa[13] suggested that regular calcium carbonate ($CaCO_3$) is a safe and viable drug carrier for the intranasal delivery of various drugs including insulin. It is shown in a separate report that compared with liquid formulation, regular $CaCO_3$ powder as a carrier improves the intranasal bioavailability of elcatonin.[14]

In the present study, porous spherical (PS-) $CaCO_3$ having a characteristic particle shape, as an intranasal drug carrier for the treatment of postprandial hyperglycemia in diabetes, was evaluated in cynomolgus monkeys and human volunteers, and compared with regular $CaCO_3$.

## MATERIALS AND METHODS

### *Materials*

Human recombinant insulin (fast-acting, water-insoluble product) for human intranasal administration (potency 26.7 U/mg) and insulin for animal nasal administration (potency 28.7 U/mg) were purchased from Novo-Nordisk A/S (Bagsvaerd, Denmark) and Intergen Ltd. (New York, NY), respectively. Human recombinant insulin solution (fast-acting, water-soluble product) used for subcutaneous study [Novolin® R injection (potency 40 U/mL)] was purchased from Novo-Nordisk A/S. Regular $CaCO_3$ was purchased from Sumida Shokai (Tokyo, Japan). PS-$CaCO_3$ was purchased from Tsutsumi Techno Planning (Tokyo). PS-$CaCO_3$ has a characteristic spherical particle shape with a large number of porous, sliver crystals produced by a specialized manufacturing process. Other chemicals and reagents were purchased commercially.

### *Animals*

Male cynomolgus monkeys (Shin Nippon Industries, Kagoshima, Japan), weighing 3.7–4.2 kg, were used. Prior to the experiment the animals were fasted overnight; however, water was available ad libitum. The animals were rested for at least 2 weeks between experiments. The animal study was performed after receiving approval from the Ethics Committee of Shin Nippon Biomedical Laboratories Ltd.

### *Volunteers*

Sixty-five healthy male volunteers 21–42 years old participated in the study after returning informed consent forms. The protocol for the study was approved by the Ethics Committee of CPC Clinic. Each subject was dosed after a 12-h overnight fast.

### *Characterization of calcium carriers*

The shape and surface characteristics of each calcium carrier were investigated by scanning electron microscopy (model JSM-5200, JEOL Ltd., Tokyo). The specific surface area of each calcium carrier was measured by Brunauer-

Emmett-Teller and nitrogen gas adsorption methods.

*Preparation of the insulin formulation*

Regular $CaCO_3$ and PS-$CaCO_3$ within the particle size range of 20–32 $\mu$m were divided by sieving. The resultant material was mixed well for 5 min after the addition of insulin powder and purified water, and the final insulin formulation was prepared after freeze-drying. Mixtures of 32 mg (for monkeys) and 32 or 48 mg (for humans) were loaded into HPMC capsules (Shionogi Qualicaps Ltd., Nara, Japan). Six types of intranasal insulin formulation were prepared: 16 U/32 mg of insulin/regular $CaCO_3$ and 16 U/32 mg of insulin/PS-$CaCO_3$ (for monkeys); 16 U/32 mg of insulin/regular $CaCO_3$ and 16 U/32 mg, 48 U/48 mg, and 64 U/48 mg of insulin/PS-$CaCO_3$ (for humans). Insulin levels in the capsules were confirmed to be between 95% and 105% and to be stable for at least 6 months after preparation, at 25°C and 60% humidity, by a high-performance liquid chromatography determination of human insulin based on USP.

*Toxicity study*

Before the clinical study, toxicity studies of our intranasal insulin formulation, including urine and blood tests, testicular toxicity tests, mutagenicity tests, and local irritation tests (nasal mucosa), etc., were performed with repeated administrations to cynomolgus monkeys. Insulin was administered intranasally once a day for 4 weeks at doses of 0/0 (air control), 0/25, 16/16, 20/20, and 25/25 (U of insulin/mg of calcium carrier). In local irritation tests of nasal mucosa, nasal epithelia (olfactory and respiratory area) were observed by optical microscopy under hematoxylin and eosin staining. During and/or after repeated administrations over 4 weeks, we observed body weights and performed urine tests (color, pH, glucose, ketone body, bilirubin, urine occult blood, urobilinogen, protein, etc.), hematology tests (erythrocyte count, leukocyte count, hematocrit value, hemoglobin concentration, blood platelet count, mean corpuscular volume, mean corpuscular hemoglobin, etc.), and serum biochemistry tests (aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, creatine phosphokinase, total bilirubin, total protein, etc.).

*Device for intranasal administration*

Each insulin formulation was administered intranasally with a nasal spray device (Jetlizer®, Unisia Jecs Corp., Gunma, Japan). This hand-operated device disperses the powder as widely as possible onto the nasal mucosa. A capsule containing the appropriate dose is loaded into the device. Thin needles built into the device pierce the top and bottom of the capsule. Pressurized air is then forced through the capsule by pressing an air pump of the device. The insulin formulation is gathered up by the pressurized airflow and released from the capsule. It was confirmed that >98% of the contents of the capsules was released.

*Insulin administration and blood samples*

*Subcutaneous administration*. In the animal study, insulin solution was administered subcutaneously in the neck at a dose level of 0.5 U per monkey. Blood (1.5 mL) was drawn from the femoral vein 0, 10, 20, 30, 40, 60, 120, and 240 min after administration.

In the clinical study, insulin solution was administered subcutaneously in the abdomen at a dose level of 4.0 U per human subject. Blood (4.0 mL) was drawn from the antecubital vein 0, 5, 15, 30, 45, 60, 90, 120, 180, 240, and 360 min after administration.

*Intranasal administration*. In the animal study, each insulin formulation was administered intranasally at a dose level of 16 U per monkey using the exclusive device. Blood (1.5 mL) was drawn from the femoral vein 0, 10, 20, 30, 40, 60, 120, and 240 min after administration.

In the clinical study, each insulin formulation was administered at dose levels of 16, 48, and 64 U per human subject using the device. Blood (4.0 mL) was drawn from the antecubital vein 0, 5, 15, 30, 45, 60, 90, 120, 180, 240, and 360 min after administration.

## *Assay method*

Serum was separated by centrifugation at 4°C and 1,600 *g*, and then stored at −20°C

until analysis. Serum glucose levels were determined by the glucokinase/glucose 6-phosphate dehydrogenase method (Iatron Laboratories, Ltd., Tokyo). Serum insulin levels were determined by the enzyme-linked immunoassay method, using an Abbott IMx analyzer (Abbott Laboratories, North Chicago, IL). The coefficient of variance of the standard curve ranged from 3.09% to 3.21%. The squared correlation coefficient was >0.997.

*Pharmacokinetic parameters*

The maximum serum concentration ($C_{max}$) and the time to reach $C_{max}$ ($T_{max}$) were recorded for each observed serum insulin concentration profile. The area under the serum concentration–time curve (AUC) was calculated by the trapezoidal method.

The relative bioavailability (BA) of intranasal insulin delivery was calculated in comparison with subcutaneous administration and was expressed as $BA = Dose_{sc}/Dose_{nasal} \times AUC_{nasal}/AUC_{sc} \times 100$. The relative BA in monkey studies was calculated using each AUC for 0–4 h after dosing and was expressed as $BA_{0-4\ h}$. The relative BA in human studies was calculated using each AUC for 0–1 h or 0–6 h after dosing to evaluate the relation between the effective absorbing period for the intranasal administration and BA, and was expressed as $BA_{0-1\ h}$ and $BA_{0-6\ h}$, respectively.

*Statistical analysis*

Statistical significance was evaluated by Student's $t$ test. Results were expressed as the mean ± SD of at least six experiments.

## RESULTS

*Characterization of calcium carriers*

Electron micrographs of regular $CaCO_3$ and PS-$CaCO_3$ are shown in Figure 1. The particle shape and surface structure of regular $CaCO_3$ are of a latticed cuboid with smooth surfaces. In contrast, those of PS-$CaCO_3$ are spherical with a rough, porous surface. As shown in the differences in structural characteristics, the measured specific surface area of PS-$CaCO_3$ (1.99 $m^2/g$) was approximately 15 times greater than that of regular $CaCO_3$ (0.12 $m^2/g$). The difference between the structural characteristics of the two calcium carriers was thought to influence their mutual contact properties with the insulin and nasal mucosa.

*Toxicity study*

In the repeated intranasal administrations for 4 weeks, local toxicity to the nasal epithelium (olfactory and respiratory areas) was not observed even when insulin was administered intranasally at a maximum dose level of 25 U. In urine, hematology, and serum biochemistry

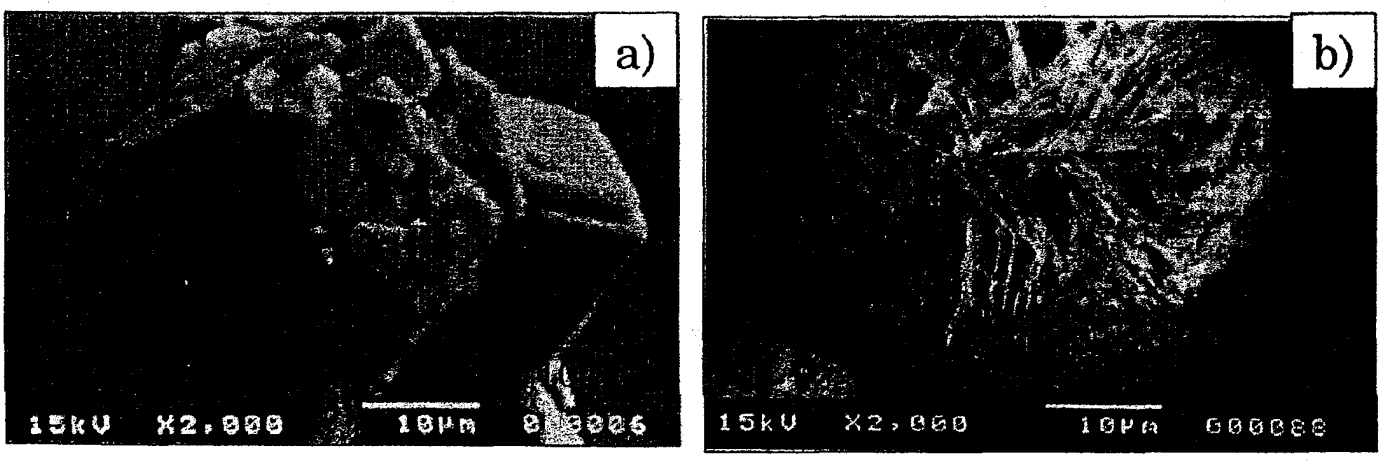


FIG. 1. Electron micrographs of calcium carriers: (a) regular $CaCO_3$ at ×2,000 magnification and (b) PS-$CaCO_3$ at ×2,000 magnification.

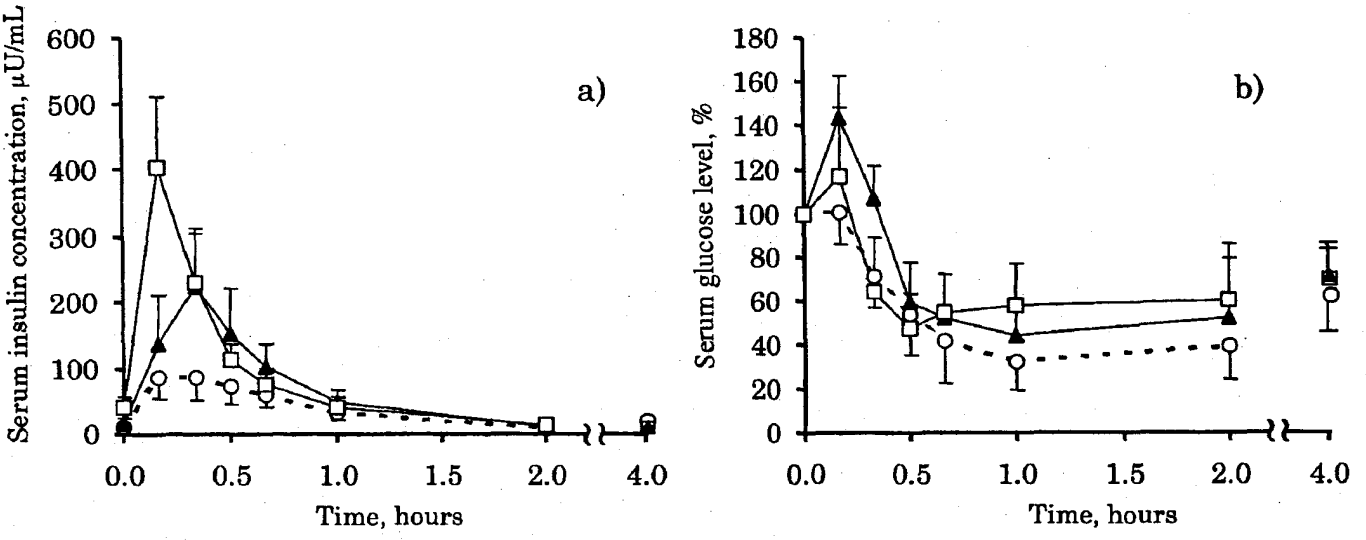


FIG. 2. Serum insulin concentration curves (a) and serum glucose level curves (b) after subcutaneous and intranasal administration in monkeys. Doses of insulin administered subcutaneously and intranasally were 0.5 and 16 U per monkey, respectively. Results are expressed as mean ± SD of six experiments. Serum glucose levels are expressed as the ratio of serum glucose concentration to the initial concentration. Each insulin formulation is expressed as follows: (open circle) subcutaneous formulation; (solid triangle) intranasal formulation with regular $CaCO_3$; (open square) intranasal formulation with PS-$CaCO_3$.

tests, no apparent differences were also observed between the control animals and those treated with the calcium carrier with and without insulin for 4 weeks. In the other toxicity studies, no apparent differences were also observed in comparison with control animals. These suggested safety of our intranasal insulin delivery system with the calcium carrier.

*Animal study*

Figure 2 shows the average serum concentration profiles of insulin and glucose after subcutaneous or nasal administration with regular $CaCO_3$ and nasal administration with PS-$CaCO_3$. The corresponding pharmacokinetic parameters are summarized in Table 1. The serum insulin level after intranasal insulin delivery with PS-$CaCO_3$ showed a higher $C_{max}$ (403.5 μU/mL) and shorter $T_{max}$ (0.167 h) when compared with intranasal delivery with regular $CaCO_3$. $BA_{0-4\ h}$ of insulin containing regular $CaCO_3$ and PS-$CaCO_3$ was found to be 5.7% and 7.0%, respectively. The serum glucose level after intranasal delivery with PS-$CaCO_3$ was reduced rapidly to its nadir at 0.5 h compared with nasal administration with regular $CaCO_3$, reflecting the difference in the insulin absorption rates. The initial increase of serum glucose level immediately after dosing was deduced to result from the stress of administration (not observed in human studies).

TABLE 1. PHARMACOKINETIC PARAMETERS OF INSULIN (MONKEY)

| *Route, formulation* (n) | *Dose* (*U per monkey*) | $T_{max}$ (*h*) | $C_{max}$ (*μU/mL*) | $AUC_{0-4\ h}$ (*μU • h/mL*) | *Relative* $BA_{0-4\ h}$ (%) | *Glucose nadir level* (%) |
|---|---|---|---|---|---|---|
| Subcutaneous | | | | | | |
| Solution (6) | 0.5 | 0.25 ± 0.09 | 89.1 ± 33.1 | 91.7 ± 18.5 | — | 28.5 ± 13.0 |
| Intranasal | | | | | | |
| Regular $CaCO_3$ (6) | 16 | 0.33 ± 0.00 | 223.0 ± 79.7 | 165.0 ± 57.6 | 5.7 ± 2.0 | 38.7 ± 17.7 |
| PS-$CaCO_3$ (6) | 16 | 0.17 ± 0.00 | 403.5 ± 106.8[1] | 206.8 ± 43.4 | 7.0 ± 1.5 | 45.6 ± 14.8 |

Results are expressed as mean ± SD.
[1] $p < 0.01$ compared with $CaCO_3$.

*Clinical study*

The intranasal delivery of insulin with regular $CaCO_3$ and PS-$CaCO_3$ was investigated in healthy human volunteers. Figure 3a shows the average serum concentration profiles of insulin and serum glucose levels after subcutaneous injection and intranasal administration (16 IU per subject) with regular $CaCO_3$ and with PS-$CaCO_3$. The corresponding pharmacokinetic parameters are summarized in Table 2. Insulin, after intranasal administration using both calcium carriers, showed rapid absorption compared with subcutaneous injection. Like with monkeys, in human subjects the absorption behavior of insulin in intranasal delivery using PS-$CaCO_3$ showed a higher $C_{max}$ (17.2 $\mu$U/mL), although the AUC was equivalent (24.2 $\mu$U • h/mL) when compared with that of regular $CaCO_3$. Figure 3b shows serum glucose levels after intravenous delivery with each calcium carrier at a dose level of 16 IU per subject. In particular, the serum glucose level after intranasal delivery with PS-$CaCO_3$ was reduced rapidly compared with intranasal delivery with regular $CaCO_3$ and subcutaneous administration, reflecting the difference in absorption rates.

Insulin was administered intranasally with PS-$CaCO_3$, which produced a faster absorption rate, at dose levels of 16, 48, and 64 U per subject (Fig. 4a). Insulin absorption after intranasal delivery with PS-$CaCO_3$ increased dose-dependently, maintaining the high absorption rate. $BA_{0-1\,h}$ at each dose remained at the same level (approximately 10%), although $BA_{0-6\,h}$ decreased as the dose increased (Table 2). Figure 4b shows serum glucose levels after intranasal delivery with PS-$CaCO_3$ at insulin dose levels of 16, 48, and 64 U per subject. Serum glucose levels reduced dose-dependently and rapidly. Each serum glucose level reached its nadir within 0.75 h of dosing, and its duration was brief. When insulin was administered intranasally with PS-$CaCO_3$ at a dose level of 64 U per subject, the serum glucose level reached a nadir that was almost equal to that observed after subcutaneous injection (4 U per subject).

## DISCUSSION

The nasal route for systemic insulin delivery has received wide attention as an available alternative to the invasive parenteral route of peptide and protein drugs.[2,15,16] The intranasal delivery of peptides and protein drugs as typified by insulin simplifies self-medication, lead-

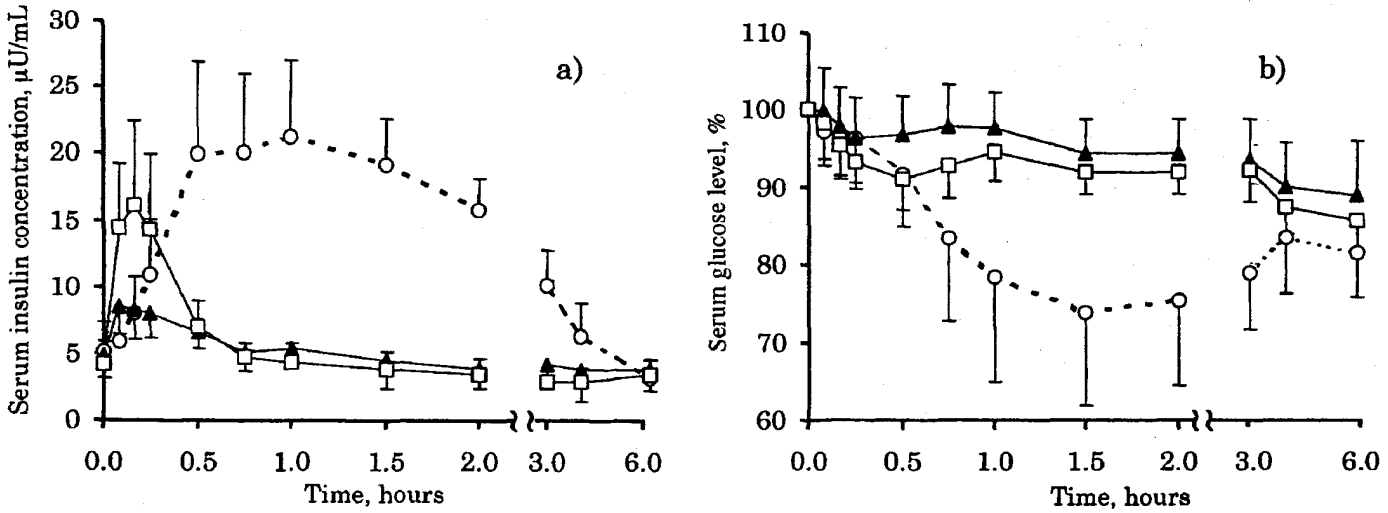


**FIG. 3.** Serum insulin concentration curves (**a**) and serum glucose level curves (**b**) after subcutaneous and intranasal administration with each calcium carrier in humans. Doses of insulin administered subcutaneously and intranasally were 4 and 16 U per subject, respectively. Results are expressed as mean ± SD of more than nine experiments. Serum glucose levels are expressed as the ratio of serum glucose concentration to the initial concentration. Each insulin formulation is expressed as follows: (open circle) subcutaneous formulation; (solid triangle) intranasal formulation with regular $CaCO_3$; (open square) intranasal formulation with PS-$CaCO_3$.

TABLE 2. PHARMACOKINETIC PARAMETERS OF INSULIN (HUMAN)

| *Route, formulation* (n) | *Dose* (U per subject) | $T_{max}$ (h) | $C_{max}$ (μU/mL) | (μU • h/mL) | | Relative (%) | | *Glucose nadir level* (%) |
|---|---|---|---|---|---|---|---|---|
| | | | | $AUC_{0-1\ h}$ | $AUC_{0-6\ h}$ | $BA_{0-1\ h}$ | $BA_{0-6\ h}$ | |
| Subcutaneous | | | | | | | | |
| Solution (23) | 4 | 0.97 ± 0.44 | 22.7 ± 6.0 | 15.8 ± 4.4 | 64.0 ± 8.3 | — | — | 72.0 ± 11.6 |
| Intranasal | | | | | | | | |
| Regular $CaCO_3$ (12) | 16 | 0.16 ± 0.07 | 9.2 ± 2.2 | 6.5 ± 1.1 | 25.9 ± 8.5 | 10.3 ± 1.7 | 10.1 ± 3.3 | 88.4 ± 4.7 |
| PS-$CaCO_3$ (9) | 16 | 0.13 ± 0.06 | 17.2 ± 5.7[1] | 8.6 ± 2.4 | 24.2 ± 5.9 | 13.6 ± 3.8 | 9.5 ± 2.3 | 84.9 ± 3.5 |
| PS-$CaCO_3$ (6) | 48 | 0.26 ± 0.12 | 37.1 ± 20.2 | 20.9 ± 13.4 | 38.2 ± 16.3 | 11.0 ± 7.0 | 5.0 ± 2.1 | 78.4 ± 11.9 |
| PS-$CaCO_3$ (6) | 64 | 0.19 ± 0.04 | 47.5 ± 18.8 | 23.9 ± 5.9 | 46.1 ± 3.9 | 9.5 ± 2.3 | 4.5 ± 0.4 | 72.8 ± 10.7 |

Results are expressed as mean ± SD.

[1] $p < 0.01$ compared with $CaCO_3$ (at 16 U per subject dose level).

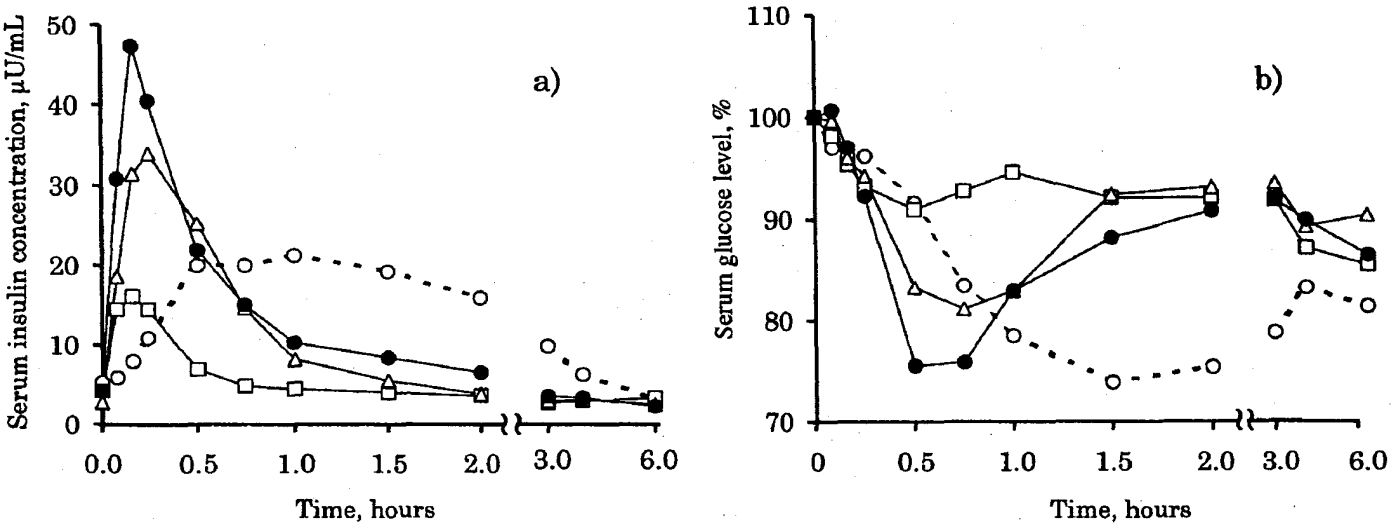


FIG. 4. Serum insulin concentration curves (a) and serum glucose level curves (b) after intranasal administration with PS-$CaCO_3$ at various insulin dose levels in humans. Doses of insulin administered intranasally were 16, 48, and 64 U per subject. Results are expressed as mean ± SD of more than six experiments. Serum glucose levels are expressed as the ratio of serum glucose concentration to the initial concentration. Each insulin formulation is expressed as follows: (open circle) subcutaneously (4 U per subject); (open square) PS-$CaCO_3$ (16 U per subject); (open triangle) PS-$CaCO_3$ (48 U per subject); (solid circle) PS-$CaCO_3$ (64 U per subject).

ing to improved compliance with patients' medical regimens. Most attempts at systemic intranasal delivery have used absorption enhancers because of poor permeability via the nasal mucosa.[11,12] The most important issue in implementing a systemic intranasal drug delivery system for clinical use is the use of a safe and promising drug carrier and/or additives to enable adequate absorbability. Yanagawa[13] suggested that regular $CaCO_3$ is a safe and available drug carrier for the intranasal delivery of various drugs including insulin. Recently, Ishikawa et al.[14] reported that water-insoluble regular $CaCO_3$ powder improved the nasal bioavailability of elcatonin when compared with liquid formulations. They explained that the mechanism of intranasal bioavailability improved by insoluble regular $CaCO_3$ powder was principally achieved by the prolongation of the residence time in the nasal cavity.

We found that PS-$CaCO_3$ is a more promising intranasal carrier for insulin, enabling effective absorption behavior for the treatment of postprandial hyperglycemia in diabetes when compared with regular $CaCO_3$ in both animals and healthy humans. To improve the intranasal insulin absorption, there are several strategies, including the wider distribution of the formulation in the nasal cavity, the greater affinity between the nasal mucosa layer and the formulation, the prolongation of nasal residence time, and the protection of insulin from enzymatic degradation. Effects of PS-$CaCO_3$ on a more effective absorption behavior of insulin were concluded to be the result of a greater affinity between the nasal mucosa layer and PS-$CaCO_3$, which is closely related to its structural characteristics, in addition to the wider distribution of the formulation in the nasal cavity achieved by the administration device.

In clinical studies, $BA_{0-1\,h}$ at each dose maintained the same level, although $BA_{0-6\,h}$ decreased as the dose increased when insulin was administered intranasally with PS-$CaCO_3$ at various dose levels. This result strongly suggested that nasal absorption of insulin was dependent on residence time in the nasal cavity. As shown in the constant $BA_{0-1\,h}$, insulin administered intranasally was absorbed dose-proportionally, while the formulation remained in the nasal cavity. However, once the formulation was removed from the nasal cavity, insulin absorption declined as shown by $BA_{0-6\,h}$. It was suggested that intranasal insulin delivery with PS-$CaCO_3$ is a viable system,

promising constant absorbability and absorption behavior of insulin while the formulation is present in the nasal cavity.

The serum glucose level after intranasal administration with PS-$CaCO_3$ was reduced rapidly in comparison with subcutaneous injection, reflecting the difference on insulin absorption rates. It is suggested that this fast action in the serum glucose level enables insulin medication immediately before or after meals. Furthermore, the short action of the pharmacological effect produced by intranasal insulin delivery suggests a reduced risk of a hypoglycemic side effect due to an excessive duration of serum insulin concentration.

In conclusion, PS-$CaCO_3$ for intranasal insulin delivery is thought to be a promising and safe carrier that can produce a more effective insulin absorption behavior similar to endogenous postprandial insulin secretion in healthy humans. To obtain strong and convincing evidence of the safety of our system, however, we have to implement an additional longer-term toxicity study. We believe that our intranasal insulin delivery system enabling a rapid and short-acting pharmacological effect against postprandial hyperglycemia will be more beneficial than pulmonary insulin delivery systems in the treatment of diabetes.

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# PUBLISHED LITERATURE

(3)

*ARTICLES ORIGINAUX*

# EFFECT OF INSULIN CONCENTRATION ON BIOAVAILABILITY DURING NASAL SPRAY ADMINISTRATION

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VALENSI P., ZIRINIS Ph., NICOLAS P., PERRET G., SANDRE-BANON D., ATTALI J.R. – Effect of insulin concentration on bioavailability during nasal spray administration.
**Path Biol,** 1996, **44,** n° 4, 235-240.

SUMMARY: The bioavailability of rapid-acting insulin administered as a nasal spray was studied in 6 type 1 (insulin-dependent) diabetic patients. They received long-acting bovine insulin (Ultratardum 40 U/ml, Organon) as basal treatment at 8 a.m. Rapid-acting insulin was also administered at 8 a.m, then at noon and 6 p.m, subcutaneously on day 1 as a 100 U/ml solution and intranasally by aerosol spray as a 100 U/ml and 500 U/ml with 1% (w/v) 9 lauryl ether solution on day 2 and day 3 respectively. On days 2 and 3, the dose of insulin was at least nine times higher than the subcutaneous dose on day 1. Free and total plasma insulin concentrations were assayed after the noon insulin administration. The peaks of the free and total plasma insulin levels were reached earlier and the return to basal levels was obtained earlier after nasal insulin administration than after insulin injected subcutaneously. The bioavailability of nasal spray insulin versus subcutaneous insulin with a 100 U/ml insulin solution was similar to that with a 500 U/ml insulin solution : 5.14 ± 0.38 % (m ± SEM) and 4.64 ± 0.46 % according to the total plasma insulin level. This study suggests that the bioavailability of nasal spray insulin is not increased by increasing insulin concentration in our experimental conditions.

KEY-WORDS: Insulin. – Nasal administration. – Bioavailability. – Insulin-dependent diabetes.

VALENSI P., ZIRINIS Ph., NICOLAS P., PERRET G., SANDRE-BANON D., ATTALI J.R. – Effet de la concentration d'insuline administrée par spray nasal sur sa biodisponibilité. *(En Anglais).*
**Path Biol,** 1996, **44,** n° 4, 235-240.

RÉSUMÉ: La biodisponibilité de l'insuline ordinaire administrée par voie nasale a été étudiée chez 6 diabétiques insulinodépendants (type 1). Ils ont reçu une injection d'insuline lente bovine (Ultratardum 40 UI/ml, Organon) à 8 heures du matin. L'insuline ordinaire a été aussi administrée à 8 heures du matin puis à midi et à 18 heures, par voie sous-cutanée le premier jour sous forme d'une solution à 100 UI/ml et par voie nasale à l'aide d'un aérosol à la concentration de 100 UI/ml et 500 UI/ml avec une solution à 1% (w/v) de 9 lauryl ether, respectivement le second et le troisième jour. Les second et troisième jour, la dose d'insuline a été au moins 9 fois plus élevée que la dose administrée par voie sous-cutanée le premier jour. Les concentrations plasmatiques d'insuline libre et d'insuline totale ont été déterminées après l'administration d'insuline à midi. Les pics plasmatiques d'insuline libre et d'insuline totale ont été atteints plus tôt et le retour aux valeurs basales ont été obtenus plus tôt après l'administration d'insuline par voie nasale qu'après l'injection d'insuline par voie sous-cutanée. La biodisponibilité de l'insuline par spray nasal par rapport à l'insuline sous-cutanée était très voisine avec la solution d'insuline à 100 UI/ml et avec celle à 500 UI/ml: 5,14 ± 0,38 % (m ± SEM) et 4,64 ± 0,46 %, le calcul étant effectué avec le taux plasmatique d'insuline totale. Cette étude suggère que l'absorption nasale d'insuline est un phénomène saturable.

MOTS-CLÉS: Insuline. – Administration nasale. – Biodisponibilité. – Diabète insulino-dépendant.

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There is a great deal of evidence showing the influence of good metabolic control on the microvascular complications of diabetes [1]. Good control with continuous subcutaneous infusion of insulin or with multiple injections can be obtained with the help of blood glucose self-monitoring. However many patients are unwilling to undergo these treatments, and subcutaneous administration is subject to wide intra-individual variations in absorption [3]. Moreover, the kinetics of the plasma insulin level subsequent to the resorption of insulin from the subcutaneous administration site are considerably different from the kinetics of the physiological post-prandial insulin level [8].

Administration of a nasal insulin spray has been shown to achieve a plasma insulin pattern closer to the physiological one, and kinetics very similar to those observed after an intravenous injection [7, 9]. In insulin-dependent diabetic patients the use of a nasal insulin spray before a standard meal can induce a significant reduction in postprandial hyperglycemia [9].

The aim of this study was to assess the effect of two different concentrations of insulin administered as a nasal spray on its bioavailability in insulin-dependent diabetic patients.

## PATIENTS, MATERIALS AND METHODS

### Patients

Six insulin-dependent diabetic patients aged 21 to 80 years (mean age : 47.3), diabetic for 5 to 40 years (mean duration : 23) (table I), were studied after obtaining their informed consent. All were diagnosed as type I diabetics as there was no rise in plasma C peptide after an iv injection of glucagon. One of them was smoker.

### Materials

Forty units/ml bovine insulin zinc crystalline suspension (ULTRATARDUM, Organon, France) was the basal long-acting insulin used in this study. Freeze-dried porcine insulin, 500 U per vial (ENDOPANCRINE 100, Organon, France) was used as the rapid-acting insulin either as a 100 U/ml solution in phosphate buffer pH 7.4 for subcutaneous administration, or as 500 U/ml and 100 U/ml solutions with 1 % (w/v) 9 lauryl ether (P.V.O. Int., Boonton N.J., USA) as an absorption enhancer in the same buffer for intranasal treatment by spray. Commercially available standard spray dispensers for nasal application calibrated to deliver 90 µl of solution per spray were used for this treatment.

### Protocol

The study was carried out on three consecutive days following the hospitalisation of the 6 patients. Blood glucose control was obtained by associating 40 U/ml long-acting insulin, injected subcutaneously at 8 a.m each day, with a 100 U/ml rapid-acting insulin solution, injected subcutaneously the first day (D1), 30 min before each meal, at 8 a.m., noon and 6 p.m. On the second day (D2) the long-acting insulin was given at 8 a.m., with 100 U/ml rapid-acting insulin administered as a nasal spray before each meal (8 a.m., noon and 6 p.m), each dose being at least 9 times that given subcutaneously on D1 (table I). On the third day (D3) the long-acting insulin was again administered at the same dose but with 500 U/ml rapid insulin given before each meal as a nasal spray with each dose at least 9 times that given on D1. These doses were then adjusted to the blood glucose level on the afternoon of D2 (table II).

Throughout the three days of the study the number of calories and the carbohydrate, lipid and protein distribution remained unchanged.

On each test-day an indwelling teflon microcatheter was inserted into a superficial vein of the forearm 15 min before the noon insulin administration, at 11 : 45, and the patients had lunch at 12:30.

Venous samples were taken at 0 min (just before the insulin administration) and every 10 min during the first hour, every 30 min for 2 hours, and finally every hour for 3 hours, to assay blood sugar, and total and free plasma insulin concentration.

Total insulin was assayed by RIA with the RIA gnost kit, bottle-assay (Behring Institute, Marburg, West Germany). Free insulin concentration was assayed with the same kit, with PEG added in order to precipitate insulin-insulin antibody complexes.

The maximum decrease of glycemia and the maximum increase of free insulin were calculated for each subject.

The area under the insulin-time curve (AUC) was calculated from the plasma insulin values obtained at each experimental time point (from 0 to 360 min) using the trapezoidal rule.

TABLE I. – **Clinical characteristics of the patients.**

TABLEAU I. – Caractéristiques cliniques des patients.

| Patients | Sex | Age (years) | Body mass index (kg/m²) | Smoker | Diabetes duration (years) |
|---|---|---|---|---|---|
| 1 | F | 33 | 21.6 | No | 5 |
| 2 | F | 66 | 26.9 | No | 32 |
| 3 | M | 51 | 26.8 | No | 24 |
| 4 | M | 77 | 20.6 | No | 40 |
| 5 | F | 36 | 23.1 | No | 27 |
| 6 | F | 21 | 26.1 | Yes | 10 |

TABLE II. – **Doses of insulin (IU) administered at noon on the three days of the protocol: subcutaneously on D1, intranasally on D2 and on D3.**

TABLEAU II. – Doses d'insuline (IU) administrées à midi lors des trois jours du protocole : par voie sous-cutanée à J1, intranasale à J2 et J3.

| Patients | D1 | D2 | D3 | D3/D2 |
|---|---|---|---|---|
| 1 | 8 | 135 | 225 | 1.67 |
| 2 | 10 | 180 | 225 | 1.25 |
| 3 | 10 | 135 | 162 | 1.20 |
| 4 | 10 | 144 | 180 | 1.25 |
| 5 | 16 | 216 | 315 | 1.46 |
| 6 | 10 | 135 | 135 | 1.00 |

The percent bioavailability of the intranasal insulin route was determined according to rapid-acting insulin administered subcutaneously at noon on D1 according to the following equation:

$$\frac{\text{AUC insulin, nasal spray}}{\text{AUC insulin SC}} \times \frac{\text{Dose units, SC}}{\text{Dose units, nasal spray}} \times 100 = \%\ \text{bioavailability}$$

Results were expressed as the means ± SEM.

Statistical comparisons were made with the two-way analysis of variance, and a $p < 0.05$ value was considered to reflect a significant difference.

## RESULTS

Blood glucose at 0 min on D2 and D3 was higher than on D1, probably because of the insufficient effect on blood glucose of the insulin spray administered at 8 a.m. The blood glucose fall on D1 was very marked but gradual. On D2 the 100 U insulin spray induced a less marked fall in blood glucose with a nadir reached 20 min after the beginning of the meal (mean decrease: 11.0%, vs 24.2%); blood glucose then rose rapidly. On D3 after the 500 U insulin spray a very slight reduction (−6.8%, 20 min after the beginning of the meal) in blood glucose was obtained. On D2 and D3 the post-prandial rise was slight and did not exceed 4.7 mmol/l on an average, with maximal post-prandial blood glucose being 21.1 mmol/l (fig. 1). The maximal decrease of glycemia was lower on D2 and D3 than on D1, ($F = 3.033$, $p = 0.08$) (table III).

The total plasma insulin level on D1 rose progressively and reached its peak value at 120 min ; then it decreased but reached its basal value only 6 hrs after the injection (fig. 2). On D2 and D3, the peak of insulin concentration was observed earlier than on D1. The decrease was then rapid and the basal value was reached within 90-120 min after insulin administration on D2 and D3 (fig.2). The free insulin time course was very similar as that for total insulin on D1, D2 and D3 (fig. 3).After the subcutaneous injection of rapid insulin (D1), the free plasma insulin level rose sharply but followed a biphasic pattern, reaching a peak 2 hrs after injection and then going back to the basal level 6 hrs after injection. On D2 and D3 the rise in the free insulin level was much quicker. The peak was reached at 30 min on D2 and 40 min on D3, i.e. at the same time as the peak of total plasma insulin. The free insulin concentration then rapidly decreased, returning to the basal level at 180 min on D2 and 120 min on D3 . The rise in the free insulin level was higher on D2 than on D3 and the mean peaks were 65 U/ml and 33 U/ml respectively (fig. 3). The maximal increase of free insulin was significantly higher on D1 than on D2 and D3 ($F = 15.476$, $p = 0.0002$) (table III).

**TABLE III. – Maximal decrease of glycemia (ΔG: mmol/l) and maximal increase of free insulin concentration (ΔFI: μIU/ml) on D1 (subcutaneous insulin injection), and D2 and D3 (intranasal insulin administration).**

TABLEAU III. – Réduction maximale de la glycémie (ΔG: mmol/l) et augmentation maximale de l'insuline libre (ΔFI: μIU/ ml) à J1 (injection sous-cutanée d'insuline) et à J2 et J3 (administration intranasale d'insuline).

| | D1 | | D2 | | D3 | |
|---|---|---|---|---|---|---|
| | ΔG | ΔFI | ΔG | ΔFI | ΔG | ΔFI |
| 1 | 2.5 | 35.1 | 1.4 | 0.41 | 0 | 0.23 |
| 2 | 3.4 | 6.2 | 0 | 0.45 | 0 | 0.57 |
| 3 | 15.6 | 10.4 | 2.0 | 4.36 | 1.8 | 1.02 |
| 4 | 11.9 | 30.0 | 6.1 | 1.67 | 2.1 | 1.67 |
| 5 | 1.5 | 13.7 | 2.6 | 0.41 | 3.8 | 0.08 |
| 6 | 5 | 30.4 | 1.9 | 1.03 | 4.1 | 1.16 |
| MEAN | 6.65 | 21.00 | 2.33 | 1.39 | 1.97 | 0.79 |
| SEM | 2.34 | 5.00 | 0.83 | 0.63 | 0.72 | 0.25 |

Calculations were made for the areas under the total plasma insulin curve, the bioavailability of intranasal insulins was 5.14 ± 0.38 % for the 100 U/ml solution (D2, range : 4.04-6.06 %) and 4.64 ± 0.46 % for the 500 U/ml solution (D3, range : 3.73-5.92 %).

No hypoglycemia occurred during the 3 days of the study. No nasal irritation or other side effects were reported by the patients.

## DISCUSSION

Considerable interest has been shown in intranasal insulin delivery as an alternative to subcutaneous injection as a mealtime therapy. The precise mechanism of peptide resorption after intranasal administration is not yet totally known, but the use of surfactants as absorption enhancers is recommended in order to obtain higher amounts of insulin in plasma. The present study measured the effect of one enhancer, 9 lauryl ether, on the resorption of two different concentrations of the same solution of insulin, administered as a nasal spray. It has been shown that 1 % (w/v) 9 lauryl ether allows better nasal insulin absorption than lower concentrations and has a more marked effect on postprandial blood sugar in type 1 diabetic patients [9]. There have been reports of nasal irritation with high concentrations of Laureth 9 but no such incidents were reported by our patients during the short two-day trials.

Insulin administered as a nasal spray usually induces a plasma insulin peak 10 to 15 minutes after administration, which is about the same period of time as that observed for intravenous administration [7, 9]. In this study we observed a free insulin peak and a total insulin peak that were both much earlier than the peak rea-

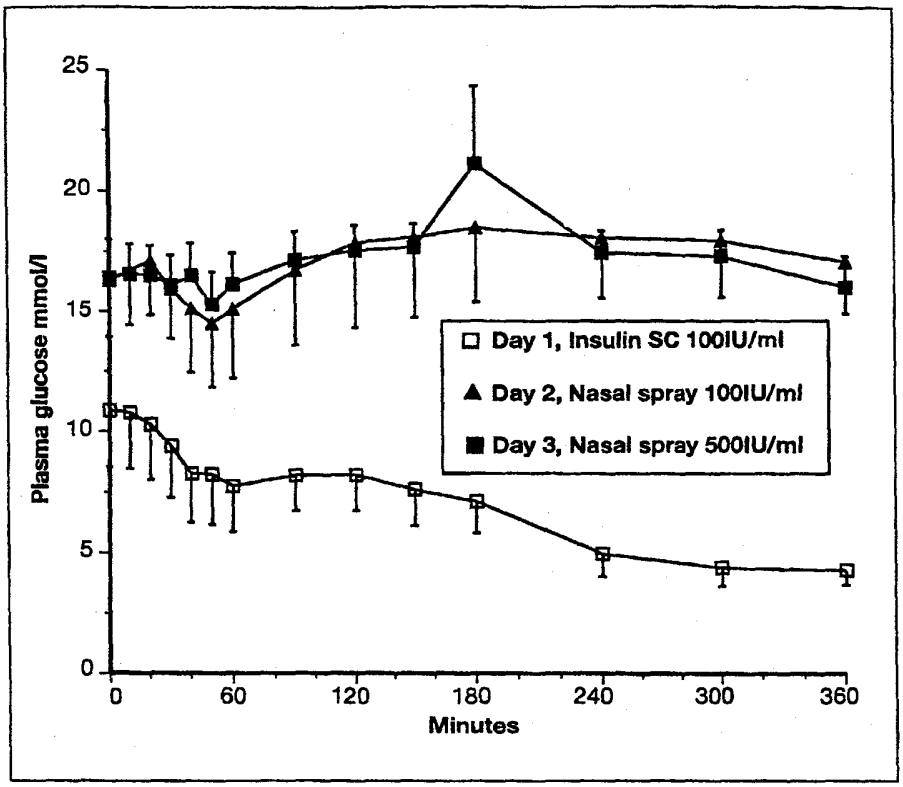


**Fig. 1. – Plasma glucose after rapid-acting insulin administered at noon subcutaneously on day 1 or intranasally as a 100 IU/ml spray on day 2 or 500 IU/ml on day 3.**

Fig. 1. – Glycémie moyenne après administration d'insuline ordinaire à midi, par voie sous-cutanée le premier jour ou par voie nasale par spray à la concentration de 100 UI/ml le second jour ou à la concentration de 500 UI/ml le troisième jour.

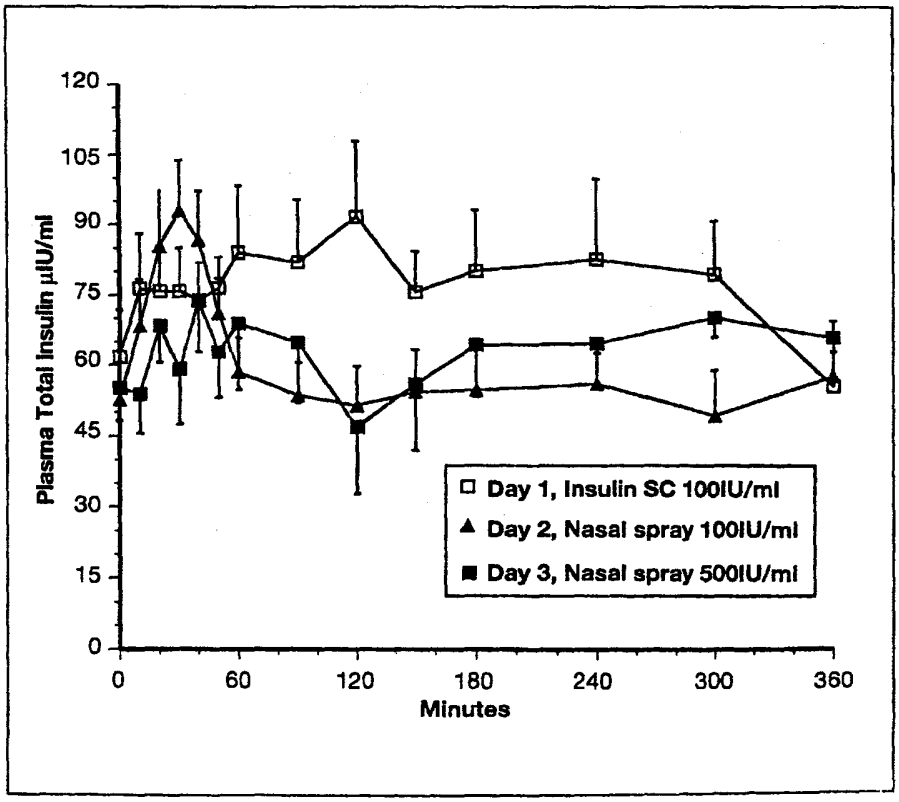


**Fig. 2. – Total plasma insulin concentration after rapid-acting insulin administration at noon. See Fig. 1.**

Fig. 2. – Concentration plasmatique d'insuline totale après administration d'insuline ordinaire à midi. Voir Fig. 1.

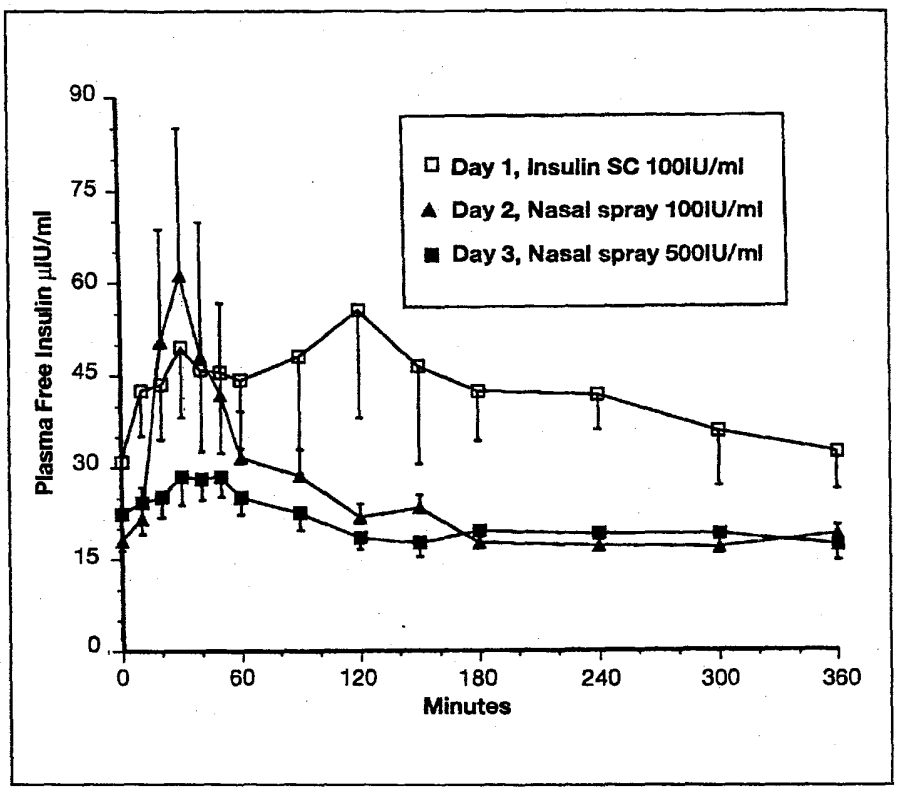


**Fig. 3. – Free plasma insulin concentration after rapid-acting insulin administration at noon. See Fig. 1.**

Fig. 3. – Concentration plasmatique d'insuline libre après administration d'insuline ordinaire à midi. Voir Fig. 1.

ched after the subcutaneous 100 U/ml insulin injection. The blood glucose nadir was here seen 50 minutes after nasal spray administration, a time period comparable to that mentioned in the literature [7, 9]. The very transient effect of nasal insulin spray has also been found in a long-term clinical study [10]. Regular porcine insulin with 0.25% 9 lauryl ether was administered intranasally for 1 month instead of the preprandial subcutaneous bolus in 10 ambulatory pump-treated IDDM patients. Nasal insulin treatment had to be withdrawn in 4 patients because of very poor glycemic control, while in the 6 other patients mean capillary glucose level before the evening meal and HbA1c were significantly increased at the end of the nasal insulin treatment.

The increase in insulinemia lasts only a very short time, like the insulinemia rise after intravenous administration and the physiological post-prandial rise [8]. With 500 U/ml insulin there was no actual drop in blood glucose but the postprandial blood glucose remained stable. This finding can be compared to the rise in free insulinemia which was markedly lower after the 500 U/ml insulin spray than with the 100 U/ml insulin spray (fig. 3) despite higher doses of insulin administered on day 3 than on day 2 (table II).

It is noteworthy that better results were obtained in the present study in terms of bioavailability following 100 U/ml nasal spray solution than with 500 U/ml, although no statistical difference was achieved. This result is similar to the better insulin resorption obtained when the amount of rapid insulin injected subcutaneously is reduced [6]. Moreover interestingly, Nolte et al. [6] report that in healthy volunteers receiving insulin pernasally with sodium taurodihydrofusidate as enhancer, a 0.35 U/kg dose of insulin gave rise to a higher bioavailability than a higher dose (0.70 U/kg). Both studies do not exclude the possibility that bioavailability may be increased by increasing the concentration of the enhancer.

Bioavailability of pernasal insulin was found here to have a fair inter-subject variability (CV: 16.8% and 21.5 % after 100 U/ml and 500 U/ml insulin concentrations respectively). However it was quite low, as already observed [5]. Moreover, we should bear in mind that 9 lauryl ether at a concentration of 1% (w/v) seems to cause nasal irritation when used over a long period of time [9].

The decrease in the plasma insulin level following the peak was far quicker after nasal spray than after subcutaneous injection. Interprandial hyperinsuline-

mia, which seems to act as an atherogenic factor, is avoided with this kinetic pattern [4].

In conclusion, this study suggests that the bioavailability of insulin administered as a nasal spray with 100 U/ml insulin solution is very similar to that found with 500 U/ml insulin solution, with 9 lauryl ether 1 % (w/v) as the absorption enhancer. Absorption enhancement of intranasally administered insulin by various adjuvants has been reported recently by several authors [2, 4, 11]. They nevertheless unanimously suggest the need of further evaluation of the intranasal route before considering it a satisfactory means for insulin delivery in the long-term treatment of diabetics.

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# Nasal insulin delivery in the chitosan solution: in vitro and in vivo studies

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**Abstract**

The effects of chitosan concentrations, osmolarity, medium and absorption enhancers in the chitosan solution on nasal insulin delivery were studied in vitro and in vivo. The penetration of insulin through the mucosa of rabbit nasal septum was investigated by measuring the transmucosal flux in vitro, while the nasal absorption of insulin in vivo was assessed by the efficiency in lowering the blood glucose levels in normal rats. It was demonstrated that increasing concentrations of chitosan up to 1.5% (w/v) caused an increase in the permeability of insulin across the nasal mucosa. Insulin given intranasally in hypo- or hyperosmotic formulation showed a higher hypoglycemic effect than insulin delivered in isoosmotic formulation. Insulin formulation in chitosan solution prepared with deionized water brought to a higher relative pharmacological bioavailability (Fr) value than that prepared with 50 mM pH 7.4 phosphate buffer. A formulation containing both 1% chitosan and 0.1% ethylenediaminetetraacetic acid (EDTA), 5% polysorbate 80 (Tween 80) or 1.2% β-cyclodextrin (β-CD) did not lead to a higher Fr than insulin formulated with 1% chitosan alone. The formulation containing both 5% hydroxypropyl-β-cyclodextrin (HP-β-CD) and 1% chitosan was more effective at reducing blood glucose levels than the formulation containing 5% HP-β-CD or 1% chitosan alone. The studies indicated that chitosan concentrations, osmolarity, medium and absorption enhancers in chitosan solution have significant effect on the insulin nasal delivery. The results of in vitro experiments were in good agreement with that of in vivo studies.


*Keywords:* Nasal delivery; Insulin; Chitosan; Permeability; Bioavailability; Rats

## 1. Introduction

Chitosan [2-amino-2-deoxy-(1 → 4)-β-D-glucopyranan] is a mucopolysaccharide obtained by the deacetylation of chitin in crustaceans such as crabs and shrimps. Chitosan is soluble in organic acid (acetic acid) or inorganic acid (hydrochloric acid) and positively charged. The chemical properties of the polymer are determined by the degree of deacetylation, molecular weight and viscosity. Studies (Paul and Garside, 2000) showed that chitosan is non-toxic and its $LD_{50}$ in mice exceeds 16 g/kg. Because of its biodegradability and biocompatibility, chitosan has been applied as a pharmaceutical excipient in oral, ocular, nasal, implant and transdermal drug delivery (Dodane and Vilivalam, 1998; Illum, 1998). Chitosan has been shown to have mucoadhesive properties because of its viscosity and interaction of the positively charged amino group with the negatively charged sites on the mucosa surface (Artursson et al., 1994; Luessen et al., 1996).

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Recent studies indicated that chitosan could enhance absorption of poorly absorbable drugs such as peptides and proteins (Luessen et al., 1996; Illum et al., 2000). The nasal delivery of chitosan was demonstrated to greatly enhance the absorption of insulin across the nasal mucosa of rats and sheep (Illum et al., 1994). In vivo evaluation in rabbits has proved that chitosan nanoparticles were able to improve the nasal absorption to a great extent than chitosan solution probably due to intensified contact of the nanoparticle with the nasal mucosa as compared to chitosan solutions (Fernandez-Urrusuno et al., 1999a,b). Later studies showed that chitosan nanoparticles were not as efficient as chitosan solution nor chitosan powder in terms of their nasal absorption promoting ability in rats and sheep (Dyer et al., 2002). Investigations have suggested that there are two effects of chitosan delivery systems on nasal mucosa. The mucoadhesive properties of the polymer can reduce the clearance rate of drugs from nasal cavity, thereby prolonging the contact time of chitosan delivery system with nasal epithelium. In addition, it has been shown that the interaction of the positively charged amino group of chitosan with the negatively charged sialic acid residues in mucus causes the transient opening the tight junctions and allows large hydrophilic compounds to be transported across the epithelium. The opening mechanism of the tight junctions has been demonstrated by a decrease in ZO-I proteins and the change in the cytoskeletal protein F-actin from a filamentous to a globular structure (Artursson et al., 1994; Schipper et al., 1997).

Most of studies utilized chitosan alone as absorption enhancer. Currently, it is not known if the combination of chitosan and other absorption enhancers, as well as some other factors could exhibits a synergistic effect in the nasal absorption of insulin. It is thought that EDTA affects the tight junctions interconnecting membrane cells by the removal of calcium and consequently increases paracellular transport (Cassidy and Tidball, 1967), and chitosan could also open the tight junctions. So it is interesting to study whether a nasal formulation containing both of these kinds of absorption enhancers could exhibit an additive or synergistic increase of the insulin absorption. Tweens with the ethylene oxide and a long hydrocarbon chain have been used to enhance the absorption of drugs in transdermal delivery systems (Breuer, 1979; Walters et al., 1987), and this kind of nonionic surfactants may penetrate into the intercellular matrix, increase the fluidity, and extract lipid components from biomembrane (Breuer, 1979; Walters et al., 1987). Cyclodextrins (CDs) could also extract the phospholipids and proteins from membrane (Shao et al., 1992). When chitosan interacts with the epithelial membrane, the tight junctions are opened, then Tweens or CDs could penetrate into the opened gaps between cells and extract the phospholipids in biomembrane. Thus, the tight junction proteins such as occludin (Furuse et al., 1993), claudin-1 and -2 (Furuse et al., 1998) are naked and may collapse after the removal of surrounding phospholipids, resulting in these fusion points untied. So the opening of the tight junctions may be strengthened by co-administration of chitosan and Tweens or CDs.

The purpose of this paper was to evaluate the effects of chitosan concentrations, osmolarity, medium and some absorption enhancers in chitosan solution on the insulin permeation across the rabbit nasal mucosa in vitro and the serum glucose concentrations after nasal administration of insulin to normal rats. Moreover, the correlation between the in vitro and in vivo studies was also investigated.

## 2. Materials and methods

### 2.1. Materials

Crystalline porcine zinc insulin (27.5 IU/mg) was purchased from Xuzhou Biochemical Company (People's Republic of China), and $^{125}$I-insulin was obtained from the China Institute of Atomic Energy. EDTA, Tween 80, β-CD and HP-β-CD were supplied by Sigma (St. Louis, MO, USA). Chitosan (non-salt form) was purchased from Qingdao Haihui Company (People's Republic of China). The molecular weight of chitosan is about 100,000 Da and the deacetylation degree is 85%. The blood glucose assay kit was a product of Zhongsheng High-Tech Bioengineering Company (People's Republic of China). All other reagents were of analytical grade.

### 2.2. Preparation of insulin formulations

Chitosan was dispersed in deionized water, and hydrochloric acid was added into the above sys-

tem under agitation until chitosan was dissolved completely. The pH of this solution was about 4.0. $^{125}$I-insulin or unlabeled insulin was dissolved in chitosan solutions to prepare stock solution. As the controls, $^{125}$I-insulin or unlabeled insulin was directly dissolved in pH 4.0 aqueous solutions without chitosan.

The concentrations of chitosan in stock solution were 0.5, 1 and 1.5% (w/v), respectively. Different amount of sodium chloride was added into the stock solution containing 1% chitosan to achieve hypoosmolarity (50 mOsm), isoosmolarity (292 mOsm) or hyperosmolarity (612 mOsm). The 50 mM pH 7.4 phosphate buffer was also used as the medium of the test solutions, besides the deionized water. EDTA (0.1%), Tween 80 (5%), β-CD (1.2%) or HP-β-CD (5%) was dissolved in the stock solution containing 1% chitosan respectively. As the controls, each of the absorption enhancers was dissolved in the insulin solution at the same concentrations, respectively without chitosan. The insulin formulations used in this studies are summarized in Table 1.

### 2.3. Tissue preparation

The rabbit nasal mucosa was prepared as described by previous studies (Carstens et al., 1993; Hosoya et al., 1994). Male Japanese white rabbits (the Experimental Animal Center of Health Science Center of Peking University) weighing 2.5–3.0 kg were used in this study. They were fasted overnight and sacrificed by air embolism. The skin around the nasal region was removed and septum made visible by removal of the lateral wall of nasal cavity. The nasal septum was carefully isolated with a scissors and placed in ice-cold Ringer's solution (NaCl 125 mM, KCl 5 mM, $CaCl_2$ 1.4 mM, $NaH_2PO_4$ 1.2 mM, $NaHCO_3$ 10 mM and D-glucose 11 mM). Then two mucosae were carefully stripped from the nasal septum using round-edged tweezers. They were immediately mounted between the two halves of Valia-Chien diffusion chamber. The effective diffusion area of the mucosa was 0.126 cm$^2$. Each chamber was filled with 5 ml of insulin preparation or Ringer's solution. Temperature was kept at 37 °C during the experiments.

Table 1
Summary of insulin formulations administered to rats

| Impacting factor | No. | Chitosan (%) | Insulin (IU/ml) | Osmolarity | Absorption enhancer |
|---|---|---|---|---|---|
| Chitosan concentration | 1 | – | 200 | Hypo | – |
| | 2 | 0.5 | 200 | Hypo | – |
| | 3 | 1.0 | 200 | Hypo | – |
| | 4 | 1.5 | 200 | Hypo | – |
| Osmolarity | 5 | 1.0 | 200 | Hypo | – |
| | 6 | 1.0 | 200 | Iso | – |
| | 7 | 1.0 | 200 | Hyper | – |
| Medium | 8 | 1.0 | 200 | Water | – |
| | 9 | 1.0 | 200 | Phosphate buffer | – |
| Absorption enhancer | 10 | 1.0 | 200 | Hypo | – |
| | 11 | – | 200 | Hypo | 0.1% EDTA |
| | 12 | 1.0 | 200 | Hypo | 0.1% EDTA |
| | 13 | – | 200 | Hypo | 5% Tween 80 |
| | 14 | 1.0 | 200 | Hypo | 5% Tween 80 |
| | 15 | – | 200 | Hypo | 1.2% β-CD |
| | 16 | 1.0 | 200 | Hypo | 1.2% β-CD |
| | 17 | – | 200 | Hypo | 5% HP-β-CD |
| | 18 | 1.0 | 200 | Hypo | 5% HP-β-CD |
| Control (physiological saline) | – | – | – | Iso | – |

No.: number of insulin formulation; hypo: hypoosmolarity; iso: isoosmolarity; hyper: hyperosmolarity.

### 2.4. In vitro permeation studies

Prior to the experiment, the diffusion chambers were filled with Ringer's solution and kept at 37 °C for 0.5 h. Then the Ringer's solution on the donor side was replaced by the $^{125}$I-insulin solutions. At the beginning, 20 μl aliquot of sample was taken from the donor (mucosal) side. During the experiment, 3 ml of sample was removed from the acceptor (serosal) side at fixed intervals (0.5, 1, 2, 3, 4 h) and the same volumes were added into the acceptor side. The samples containing $^{125}$I-insulin were determined with a γ-counter. Each study was repeated five times.

### 2.5. Calculation of the permeability coefficient

Flux data were plotted as the cumulative amount of $^{125}$I-insulin that diffused from the mucosal to the serosal of epithelium versus time. The permeability coefficient ($P$) was calculated using the following equation:

$$P = \frac{dQ/dt}{C_0 A}$$

where $dQ/dt$ represents the permeability rate, and $C_0$ stands for the initial concentration in the donor chamber, while $A$ is the effective surface area of the mucosa.

### 2.6. In vivo studies

In vivo studies were performed as earlier reported (Chandler et al., 1991). Briefly, male Sprague–Dawley rats (the Experimental Animal Center of the Health Science Center of Peking University) weighing 250–300 g were fasted overnight and anaesthetized by intraperitoneal injection of ethyl carbamate (1.35 g/kg). The rats were tracheotomised to divert the airflow from the nasal passages and aid breathing. The oesophagus was closed by ligation onto the tracheal cannula. The left carotid artery and the right external jugular vein were cannulated for blood sampling and fluid (physiological saline) replacement, respectively. The insulin preparation (200 IU/ml) were delivered through the right nostril using a PVC tube connected to a microliter syringe to give an insulin dose of 10 IU/kg. The preparation administered nasally was about 12–15 μl, depending on the weight of the rat. Blood samples (0.2 ml) were taken at various time intervals up to 5 h after administration and the total volume of blood removed from each rat was about 1.5 ml.

The samples were centrifuged to obtain serums and the blood glucose levels were determined immediately with blood glucose assay kits using the glucose oxidase method.

As a control, the physiological saline solution without insulin was also intranasally administered to the rats. Insulin solution was subcutaneously (s.c.) administered to the rats (1 IU/kg) to calculate the relative pharmacological bioavailability (Fr). Each group contains five rats.

### 2.7. Data analysis

The area above the serum glucose levels time curves (AAC) were calculated using the trapezoidal rule. The relative pharmacological bioavailability (Fr) was calculated according to the formula (Shen et al., 1999):

$$\mathrm{Fr} = \frac{\mathrm{AACi.n.} \times \mathrm{Doses.c.}}{\mathrm{AACs.c.} \times \mathrm{Dosei.n.}} \times 100\%$$

The "i.n." and "s.c." represent "intranasal" and "subcutaneous", respectively.

Student's $t$-test and ANOVA were used to determine statistical significance. Differences were considered to be significant for values of $P < 0.05$.

## 3. Results

The effects of chitosan concentrations, osmolarity of the solution, medium as well as some absorption enhancers on the insulin permeation across the rabbit nasal mucosa in vitro are shown in Figs. 1 and 2, respectively. The permeation coefficients of insulin across the rabbit nasal epithelium in vitro under different conditions are listed in Table 2.

When the concentrations of the chitosan increased from 0 to 1.5%, the amount of insulin across the mucosa increased consequently. There were significant differences in permeation coefficients between three chitosan concentrations ($P < 0.05$). It was shown that the permeability coefficient of insulin with 1.5% chitosan is almost 25-fold higher than that without chitosan. In the hypoosmotic or hyperosmotic solution, there was a significant permeability increase

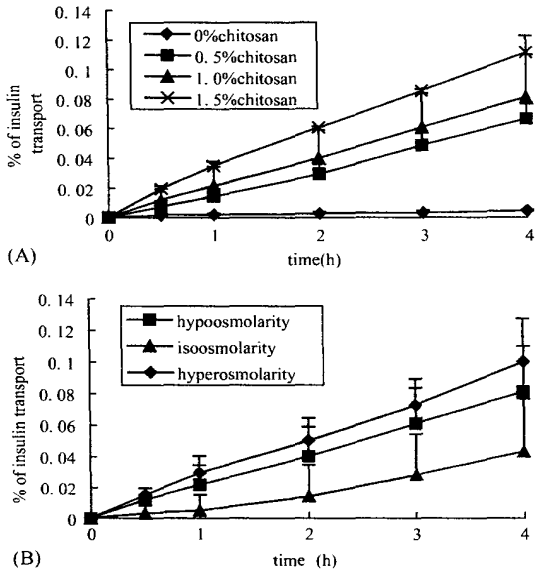


Fig. 1. Effect of concentrations (A) and osmolarity (B) of chitosan solution on the transport of insulin across the rabbit nasal membrane in vitro. Bars represent the S.D. of five experiments.

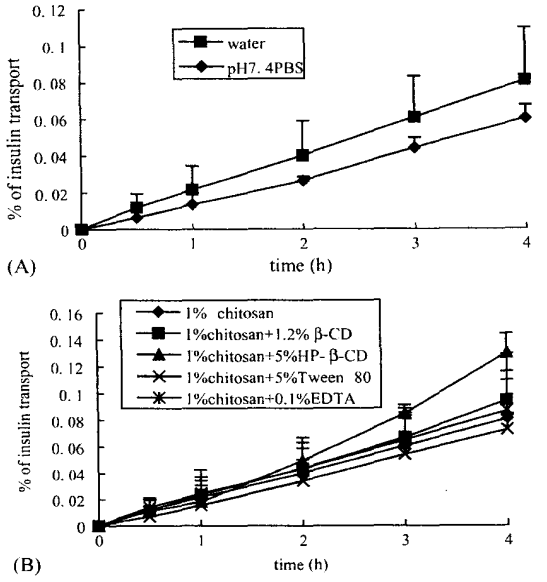


Fig. 2. Effect of medium (A) and other absorption enhancers (B) on the transport of the insulin from chitosan solution across the rabbit nasal membrane in vitro. Bars represent the S.D. of five experiments.

Table 2
The permeation coefficient ($P$) of insulin across the rabbit nasal epithelium in vitro under different conditions (mean ± S.D., $n = 5$)

| Impacting factor | Formulation | $P$ ($\times 10^{-7}$) ($cm\,s^{-1}$) |
|---|---|---|
| Chitosan concentration | 0% chitosan | 1.25 ± 0.19 |
| | 0.5% chitosan | 18.31 ± 2.75[a] |
| | 1.0% chitosan | 22.36 ± 7.93[a] |
| | 1.5% chitosan | 30.70 ± 3.11[a] |
| Osmolarity | Hypoosmolarity (1.0% chitosan) | 22.36 ± 7.93[b] |
| | Isoosmolarity (1.0% chitosan) | 11.90 ± 9.21 |
| | Hyperosmolarity (1.0% chitosan) | 27.63 ± 7.45[c] |
| Medium | Water (1.0% chitosan) | 22.36 ± 7.93 |
| | pH 7.4 phosphate buffer (1.0% chitosan) | 16.60 ± 1.98[d] |
| Absorption enhancer | 1.0% chitosan | 22.36 ± 7.93 |
| | 0.1% EDTA + 1.0% chitosan | 24.01 ± 8.13 |
| | 5% Tween 80 + 1.0% chitosan | 20.18 ± 3.08 |
| | 1.2% β-CD + 1.0% chitosan | 26.15 ± 9.55 |
| | 5% HP-β-CD + 1.0% chitosan | 35.95 ± 3.89[e] |

[a] $P < 0.05$ between 0.5, 1.0 and 1.5% chitosan concentration.
[b] $P < 0.05$ vs. isoosmolarity.
[c] $P < 0.05$ vs. isoosmolarity.
[d] $P < 0.05$ vs. water.
[e] $P < 0.05$ vs. 1.0% chitosan.

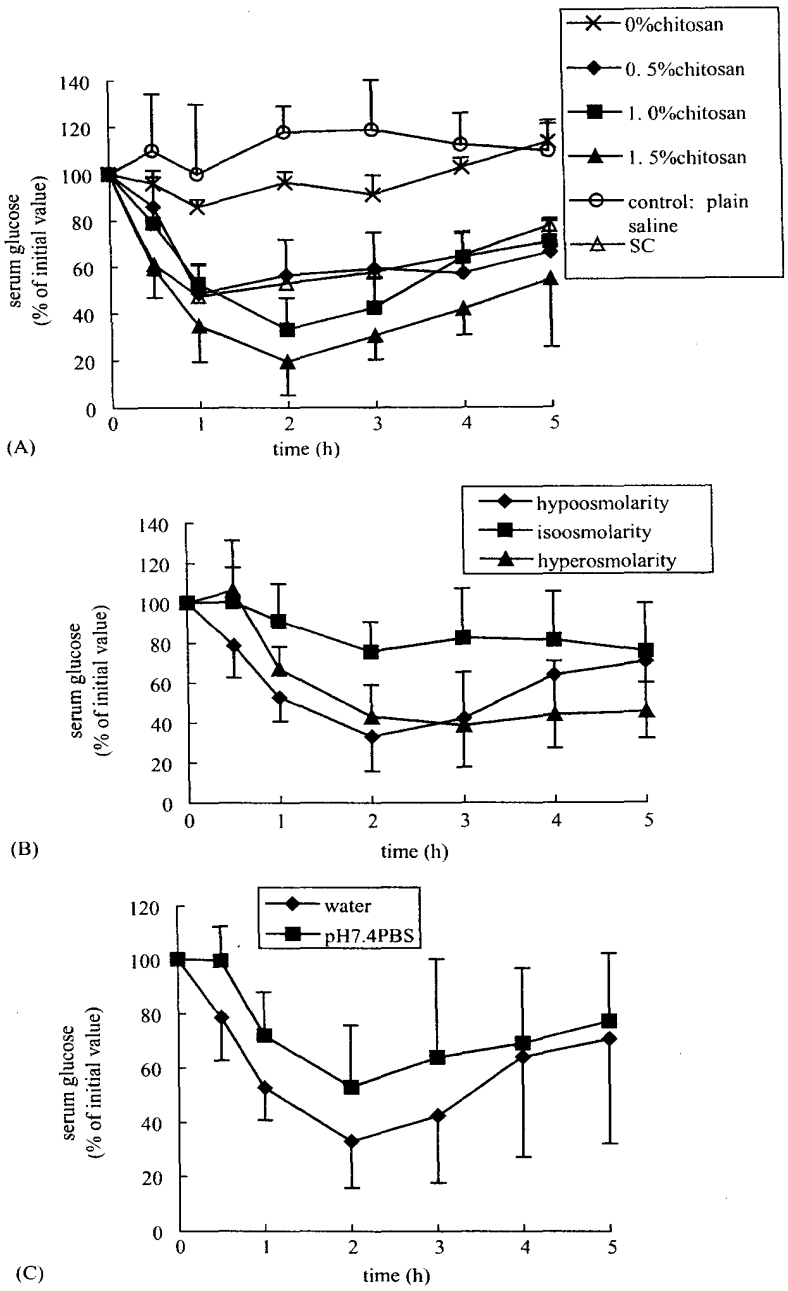


Fig. 3. Effect of concentrations (A), osmolarity (B) and medium (C) of the chitosan solution on the mean serum glucose concentrations after nasal administration of 10 IU/kg insulin to rats. Bars represent the S.D. of five experiments.

($P < 0.05$) of insulin across the mucosa, compared to that in isoosmotic solution.

An increase in the permeability coefficient of insulin in the chitosan formulation prepared with deionized water was demonstrated, as compared to that with pH 7.4 phosphate buffer. There was a remarkable increase in the permeability of insulin in the formulation containing both 5% HP-β-CD and 1% chitosan, compared to that of the control formulation containing 1% chitosan alone. No significant difference ($P > 0.05$) in the permeability coefficient was seen between the formulation containing 0.1% EDTA as well as 1% chitosan and the control formulation. The similar results with 0.1% EDTA were observed when 1.2% β-CD or 5% Tween 80 was combined with 1% chitosan.

Effects of chitosan concentrations, osmolarity of the test solutions, medium and some absorption enhancers on the mean serum glucose concentrations after nasal administration of 10 IU/kg insulin to normal rats are depicted in Figs. 3–5, respectively, while the concomitant changes in relative pharmacological bioavailability (Fr) are presented in Table 3.

As a control, nasal administration of insulin without chitosan failed to reduce the blood glucose levels, but obviously hypoglycemic effect of insulin was seen when the formulation containing different concentration of chitosan was delivered into the rat nasal as shown in Fig. 3A. The Fr for the three chitosan concentrations are significantly different ($P < 0.05$). There was an effect of the concentration increase of chitosan on the pharmacological effect of insulin (as seen in Table 3). The nadir of glucose levels were obtained 1 h after the administration of the insulin formulation containing 0.5% chitosan, while the minimum glucose levels were achieved 2 h after insulin was given together with 1 or 1.5% chitosan to the rats. The time to reach minimum glucose levels was remarkably delayed at higher concentration of chitosan compared to that at lower concentration. There were significant differences in Fr and nadirs between 1 and 1.5% chitosans ($P < 0.05$).

Insulin in isoosmotic formulation showed a relative weak effect on lowering the blood glucose contents as given in Fig. 3B. However, the administration of insulin solution, either hypo- or hyperosmolarity, resulted in a significant decrease in blood glucose concentrations ($P < 0.05$). The relative pharmacological bioavailabilities (Fr) of three insulin formulations

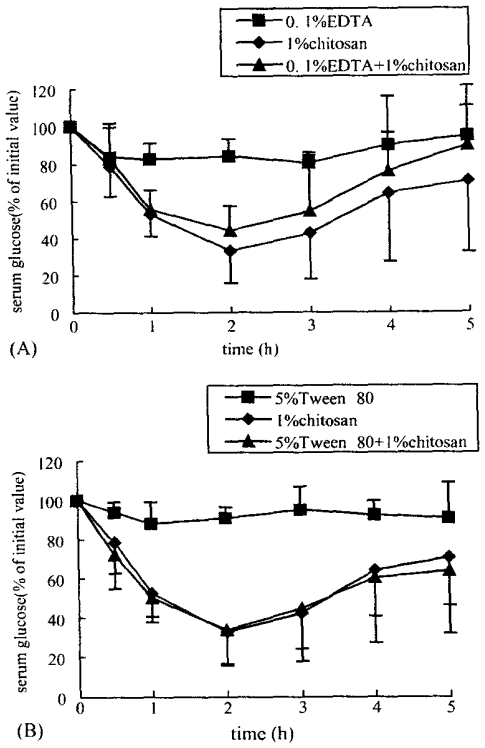


Fig. 4. Effect of EDTA or/and chitosan (A), Tween 80 or/and chitosan (B) on the mean serum glucose concentrations after nasal administration of 10 IU/kg insulin to rats. Bars represent the S.D. of five experiments.

were consistent with their permeability coefficients in vitro.

The effect of reducing blood glucose levels of the insulin formulation prepared with deionized water was more effective than that prepared with pH 7.4 phosphate buffer as illustrated in Fig. 3C, and there are significant differences between them in Fr ($P < 0.05$).

As shown in Fig. 4A, the blood glucose levels after nasal administration of 10 IU/kg insulin to rats decreased but not significant when EDTA was used as enhancer alone at the concentration of 0.1%. Nasal delivery of insulin with 1% chitosan resulted in an obvious decrease in serum glucose levels, with a minimum value of 32.95% of the initial glucose concentration at 2 h after administration. The minimal glucose levels, 43.78% of the initial value, was obtained 2 h after

Table 3
The relative pharmacological bioavailability (Fr) after nasal administration of 10 IU/kg insulin to rats under different conditions (mean ± S.D., $n = 5$)

| Impacting factor | No. | Formulation | Fr (%) |
|---|---|---|---|
| Chitosan concentration | 1 | 0% chitosan | 0.89 ± 0.63 |
| | 2 | 0.5% chitosan | 9.77 ± 3.26[a] |
| | 3 | 1.0% chitosan | 11.35 ± 5.32[a] |
| | 4 | 1.5% chitosan | 15.41 ± 5.43[a] |
| Osmolarity | 5 | Hypoosmolarity | 11.35 ± 5.32[b] |
| | 6 | Isoosmolarity | 4.09 ± 3.95 |
| | 7 | Hyperosmolarity | 11.42 ± 5.49[c] |
| Medium | 8 | Water | 11.35 ± 5.32 |
| | 9 | pH 7.4 phosphate buffer | 7.52 ± 5.69[d] |
| Absorption enhancer | 10 | 1.0% chitosan | 11.35 ± 5.32 |
| | 11 | 0.1% EDTA | 3.61 ± 1.49 |
| | 12 | 0.1% EDTA + 1.0% chitosan | 8.88 ± 4.54 |
| | 13 | 5% Tween 80 | 1.98 ± 1.72 |
| | 14 | 5% Tween 80 + 1.0% chitosan | 11.81 ± 3.91 |
| | 15 | 1.2% β-CD | 3.43 ± 2.64 |
| | 16 | 1.2% β-CD + 1.0% chitosan | 12.61 ± 7.19 |
| | 17 | 5% HP-β-CD | 7.67 ± 3.56 |
| | 18 | 5% HP-β-CD + 1.0% chitosan | 16.08 ± 3.28[e] |
| s.c. (1 IU/kg) | – | – | 100 ± 0.47 |

s.c.: subcutaneous administration; No.: number of insulin formulation (the compositions can be referred to Table 1).
[a] $P < 0.05$ between 0.5, 1.0 and 1.5% chitosan concentration.
[b] $P < 0.05$ vs. isoosmolarity.
[c] $P < 0.05$ vs. isoosmolarity.
[d] $P < 0.05$ vs. water.
[e] $P < 0.05$ vs. 1.0% chitosan.

the administration of the insulin formulation containing 1% chitosan and 0.1% EDTA. These differences were not significant ($P > 0.05$).

When insulin formulation containing 5% Tween 80 alone was delivered into the rat nasal, a slight decrease of the glucose levels was seen as shown in Fig. 4B, as compared to the formulation containing 1% chitosan. The formulation containing 5% Tween 80 and 1% chitosan was as effective as the formulation containing 1% chitosan alone, in terms of hypoglycemic effect. The serum glucose levels after administration of these two formulations fell to 33.94 and 32.95% of the initial levels, respectively, at about 2 h. There was no significant difference ($P > 0.05$) in Fr between these two samples. The decrease in serum glucose levels showed a similar pattern when insulin was administered with 1.2% β-CD or/and 1% chitosan (Fig. 5A).

The nasal administration of insulin formulation containing 5% HP-β-CD alone produced a obvious decrease in blood glucose levels, reaching minimal value of 57.62% of the initial concentration at 0.5 h as demonstrated in Fig. 5B. Insulin formulation containing 5% HP-β-CD and 1% chitosan was more efficient at lowering glucose levels than the formulation containing 1% chitosan alone. For the late two formulations, the minimum values of 21.44 and 32.95% of the initial glucose concentrations were reached, respectively, 2 h after administration. There was a significant difference ($P < 0.05$) in Fr between those two formulations.

Taking the permeability coefficient ($P$) as $X$-axis values and relative pharmacological bioavailability (Fr) as $Y$-axis values, the correlation between in vitro and in vivo evaluation of different insulin formulations was obtained, as shown in Fig. 6, with a correlation coefficient of 0.9472, suggesting a good correlation between in vitro permeation enhancing effect and in vivo pharmacological effect.

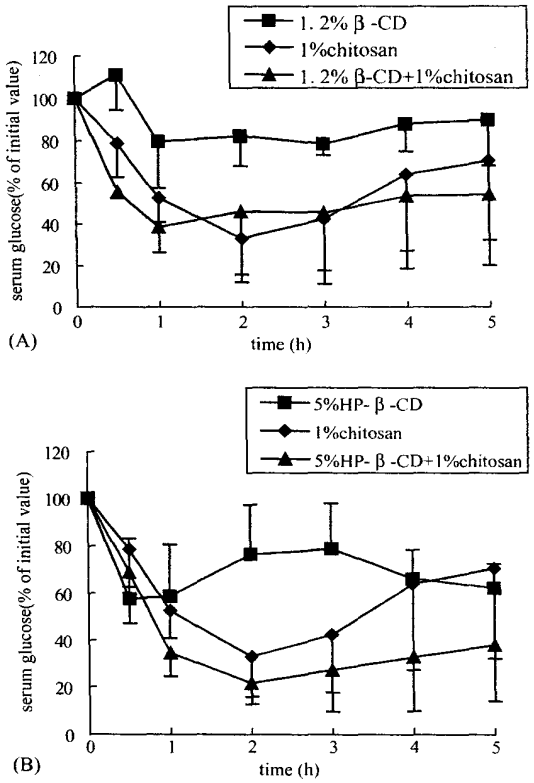


Fig. 5. Effect of β-CD or/and chitosan (A), HP-β-CD or/and chitosan (B) on the mean serum glucose concentrations after nasal administration of 10 IU/kg insulin to rats. Bars represent the S.D. of five experiments.

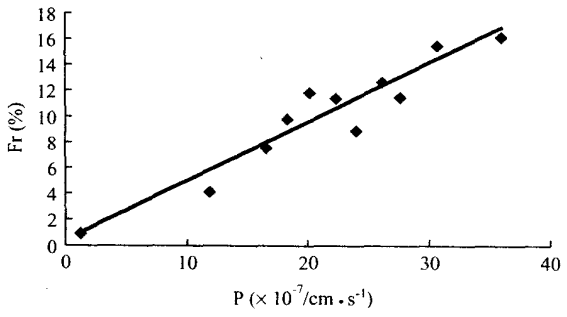


Fig. 6. The correlation between permeability coefficient ($P$) and relative pharmacological bioavailability (Fr) of different formulations.

## 4. Discussion

The influence of chitosan concentrations on the effect of insulin both in vitro and in vivo in our studies indicated that chitosan concentration is one of the impacting factors influencing the enhancement of drugs to pass through the membrane, probably due to the mucoadhesive properties and high viscosity produced by the chitosan solutions, which make the drugs stay in the nasal cavity for a long time and be cleared slowly by mucocilia from nasal mucosa. But in this rat model, the mucociliary clearance mechanism is impaired hence the mucoadhesiveness has less importance in this studies. On the other hand, chitosan may open the tight junctions between cells due to the interaction of the positively charged amino group of it with the negatively charged sialic acid residues in mucus, leading to the transport increase of large hydrophilic compounds across the epithelium, as it was mentioned in the introduction. Studies (Artursson et al., 1994) demonstrated that an increase in chitosan concentrations resulted in an increase in the permeability coefficient of $^{14}$C-mannitol with a plateau level between 0.25 and 0.5%, using a human intestinal cell line (Caco-2) as the model epithelial cell layer. It was shown by earlier studies (Lehr et al., 1992) that the minimum concentration of chitosan resulting in strong bioadhesiveness on pig intestinal mucosa was 1% (w/v). Previous studies (Illum et al., 1994) reported that the lowest plasma glucose levels were obtained after nasal delivery of insulin with 0.2% chitosan to rats, and due to the increased viscosity no increased absorption enhancement was observed when chitosan concentration was higher than 0.2%. However, in our studies the increase in chitosan concentrations from zero to 1.5% resulted in a continuous decrease in blood glucose concentrations. The minimal blood glucose level was significantly delayed for the insulin formulation containing 1 or 1.5% chitosan as compared to that containing 0.5% chitosan, which could be explained by the longer contact time between the drug and nasal membrane when higher chitosan concentration was presented. There were significant differences ($P < 0.05$) in both Fr and nadir between 1 and 1.5% chitosans in our studies. The differences between 1 and 1.5% chitosans can be explained by the following reasons. One reason is that maybe there is a correlation between the capacity of opening tight junctions

and chitosan concentrations. In addition, maybe there is also a correlation between the membrane damage and chitosan concentrations (1 and 1.5%). The previous reports (Artursson et al., 1994; Illum et al., 1994) investigated the chitosan concentrations only up to 1% and the concentrations higher 1% were not studied. The second and third reasons remain to be investigated.

In present studies, insulin delivered intranasally in the hypo- or hyperosmotic formulation was more effective in both transport increase of insulin across the rabbit nasal membrane and reducing blood glucose levels of normal rats than that in isoosmotic formulation.

The effect of hypoosmotic environment on the in vitro and in vivo studies could be possibly explained by the so-called "regulatory volume decrease (RVD) response" and "solvent drag". Firstly, the hypoosmotic environment may result in cell swelling and in most cases cell swelling will lead to cell shrinkage back to the original volume. The RVD response is triggered by the opening of volume-sensitive anion ($Cl^-$) and cation ($K^+$) channels in cell membrane accompanying with the efflux of KCl and the loss of water, and consequently promotes the transport of insulin across the biomembrane. Earlier studies (Coransanti et al., 1990) reported that a hypotonic solution cause rat hepatocytes to swell to adapt their intraosmotic pressure to tonicity of surrounding medium. These cells change their volumes and exhibit an ability to return toward their resting (isotonic) volumes. Other studies (Noach et al., 1994) demonstrated that a rapid and fully reversible drop in the transepithelial electrical resistance (TEER) and an increase in transport of fluorescein-Na or fluorescein-isothiocyanate-labeled dextran were observed when a hypotonic solution was applied at the apical side of the Caco-2 monolayers. Another mechanism called "solvent drag" shown in earlier study (Pappenheimer and Reiss, 1987) suggests that a hypotonic solution will induce fluid flow from the hypotonic to the isotonic side of a cell layer, which will enhance hydrophilic molecules to transport the epithelial cell layer.

The effect of hyperosmotic environment on the epithelial membrane seems controversial. Studies (Sakiya et al., 1981) reported that the absorption of quinine from the small intestine decreased with increasing osmotic pressure. An increase of TEER was observed when some epithelial mucosae were exposed to hyperosmotic condition, which is probably due to the collapse of the epithelial intercellular space (Madara, 1983). The same findings were also reported in other studies (Ritter et al., 1991). Earlier study showed that insulin delivered intranasally in a hyperosmotic gel system had no significant effect on reducing blood glucose levels (Pereswetoff-Morath and Edman, 1995). In contrast, the studies (Noach et al., 1994) demonstrated that a drop in TEER could be seen in Caco-2 cell test during the application of an apical hypertonic solution. The finding in our study is also interesting and the reason is not clear, probably due to the "regulatory volume increase (RVI) effect" or the lesion on the nasal epithelium caused by the hyperosmotic condition. Regulatory volume increase (RVI) could occur in response to hypertonic shrinkage of cells and is generally mediated by salt and water entry into cells (Coransanti et al., 1990). In addition, the absorption of insulin can be also driven by the difference of the osmotic pressure. In our study, this hyperosmolarity would not lead to a salt out of insulin and the insulin is stable in this environment.

The different effect of insulin formulations in different medium (deionized water and pH 7.4 phosphate buffer) could be explained by the inference that the anion ($HPO_4^{2-}$ and $H_2PO_4^-$) in the phosphate buffer would aggregate around the positively charged amino group ($-NH_3^+$) of chitosan and prevent the contact between the chitosan and negatively charged sites of nasal epithelium. It is also well known that in solution with high ionic strength, chitosan collapses because of the neutralization of the positive charges and becomes less efficient in the absorption enhancement. Even in some cases, a salting out effect could occur.

When EDTA was added into insulin solution without chitosan, zinc ions could be chelated by EDTA, leading to insulin deaggregation from hexamer to dimers as was evidenced from a circular dichroism study (Liu et al., 1991). Obviously, it is easier for the smaller molecular dimension to pass though the epithelial membrane, compared to the larger one. However, the smaller species of insulin will have higher degradation rate than the larger ones. Fig. 4A indicated that insulin released from the formulations was in its active form, showing that EDTA did not influence the stability of insulin. On the other hand, it is thought that EDTA affects the tight junctions

interconnecting membrane cells and consequently increases paracellular or pore transport (Cassidy and Tidball, 1967). But the negatively charged carboxyl groups of EDTA would interact with the positively charged amino groups of chitosan, when EDTA was mixed with chitosan, which may prevent either agent from contacting with nasal mucosa and also inhibit the deaggregation of insulin. Therefore, co-administration of EDTA and chitosan may result in a weak mutual inhibition in their abilities of absorption enhancement, which properly explained the fact in our studies that co-administration of these two absorption enhancers was not as effective as chitosan alone in lowering blood glucose levels.

With several polyoxyethylene chains and a long hydrocarbon chain, Tweens have been used to enhance the absorption of drugs in transdermal delivery system in previous studies (Breuer, 1979; Walters et al., 1987). There are two possible mechanisms by which drug absorption is increased using this kind of nonionic surfactants. Firstly, the surfactant may penetrate into the stratum corneum (SC), extract lipid components from it, disrupt the lipid arrangements, increase the fluidity, and increase the water content of the protein in the barrier, leading to a higher solubility for drugs. Secondly, the penetration of the surfactant into the SC intercellular matrix accompanied by the interaction and binding with keratin filaments may result in a disruption of the corneocyte. However, Tween 80 in the nasal delivery system in our investigations did not result in a significant absorption enhancement as compared to that in transdermal drug delivery system. The result did not reach the expected effect that Tween could work synergistically with chitosan in nasal delivery system and this is probably due to the difference between the structures of skin barrier and nasal membrane.

Cyclodextrins (CDs) could enhance nasal absorption of therapeutic compounds such as peptides and proteins. Merkus et al. (1999) have summarized the absorption enhancing effects of cyclodextrins in nasal drug delivery. But their mechanisms of absorption enhancement are not very clear. It may be attributed to some of the following reasons: (i) complexation with lipophilic penetrants, resulting in an increased water solubility for these lipophilic compounds (Schipper et al., 1990), (ii) direct epithelial membrane disruption effect by extraction of phospholipids and proteins, (iii) inhibition of proteolytic enzyme activity, (iv) dissociation of insulin oligomers. Studies (Shao et al., 1992) showed that the relative effects of various cyclodextrins on causing insulin hexamer dissociation follow the descending order of DM-β-CD (5%) > α-CD (5%) > HP-β-CD (5%) > γ-CD (5%) > β-CD (1.8%). The order of protein release rates from biomembrane follows in a descending manner: DM-β-CD (5%) > α-CD (5%) > β-CD (1.8%) > HP-β-CD (5%) > γ-CD (5%) and the rate of total phosphorus release follows the same rank order correlation as that of total protein release. β-CD and HP-β-CD are chosen as absorption promoters in this studies due to their relatively lower toxicity and irritation compared to dimethyl-β-cyclodextrin (DM-β-CD) (Yoshida et al., 1988). In present study, co-administration of 1.2% β-CD and 1% chitosan did not give rise to a higher Fr value of insulin than delivery with 1% chitosan alone. Co-administration of 5% HP-β-CD and 1% chitosan was more effective both in reducing blood glucose levels and in increasing the permeability coefficient than administration of 1% chitosan alone, suggesting that 5% HP-β-CD was more effective in the absorption enhancement for insulin than 1.2% β-CD. It was very interesting to demonstrate the synergistic effect between the two absorption-enhancing agents, HP-β-CD and chitosan, in our in vitro and in vivo studies. The result in present studies could be explained by following reasons. CDs could extract the phospholipids and proteins from membrane by forming a new lipid inclusion compartment in the aqueous phase (Shao et al., 1992). When chitosan interacts with the epithelial membrane, the tight junctions are opened, then HP-β-CD could penetrate into the opened gaps between cells and extract the phospholipids from biomembrane. Thus, the tight junction proteins such as occludin (Furuse et al., 1993), claudin-1 and -2 (Furuse et al., 1998) are naked and may collapse after the removal of surrounding phospholipids, resulting in these fusion points untied. Moreover, it has been shown that the interior of the tight junction pores are highly hydrated and negatively charged. A relative modest alteration in the relative concentration of specific species of ions within the volume of the ZO pores would result in substantial alterations in tight junction resistance leading to opening of the pore (Madara, 1989). The naked ZO pores caused by HP-β-CD could be affected more easily by the com-

position and concentration of specific species of ions in the pores. In other words, the opening of tight junctions by chitosan with HP-β-CD becomes easier than that without HP-β-CD. In addition, the dissociation of insulin and the inhibition of proteolytic enzyme activity could strengthen the absorption enhancement of insulin. So HP-β-CD could work synergistically with chitosan and the absorption enhancement of co-administration of chitosan and HP-β-CD was more effective than that of HP-β-CD or chitosan used alone. β-CD could also extract the phospholipids and release proteins from biomembrane, but the capacity of releasing proteins is greater than that of HP-β-CD (Shao et al., 1992), so the number of glycoproteins on the cell membrane exposed to β-CD is smaller than that exposed to HP-β-CD. Thus, the interaction between positively charged chitosan and negatively charged glycoproteins exposed to β-CD would be weakened to a larger extend, compared to that exposed to HP-β-CD. In addition, 1.8% β-CD was less effective in dissociating insulin hexamers than 5% HP-β-CD (Shao et al., 1992). So the enhancing effect of co-administration of chitosan and β-CD was less than that of co-use of chitosan and HP-β-CD.

This study showed that the combination of chitosan and HP-β-CD is the most effective in enhancing the absorption of insulin in nasal delivery system. The results of in vitro experiments were in good agreement with those performed in vivo, so the in vitro test could be used to evaluate the nasal delivery of insulin.

## 5. Conclusions

The results in this study indicated that the chitosan concentrations, osmolarity, medium and absorption enhancers in chitosan solution have significant effect on nasal insulin delivery. The maximum hypoglycemic effect was achieved when insulin was administered in a formulation containing both HP-β-CD and chitosan.

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